A STUDY ON MICROBIAL KERATITIS

DISSERTATION SUBMITTED FOR

BRANCH – IV – M.D . DEGREE

(MICROBIOLOGY)

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CERTIFICATE

This is to certify that the dissertation entitled **"MICROBIAL KERATITIS"** submitted by **Dr.D.SARADHA** to the Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirement for the award of M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

Director,

Institute of Microbiology,

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Madurai.

DECLARATION

I, **Dr.D. SARADHA** declare that, I carried out this work on **"MICROBIAL KERATITIS"** at the institute of Microbiology, Madurai Medical College, I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in India or abroad.

This is submitted to the Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D Degree examination in Microbiology.

Place : Madurai **Dr. D. SARADHA**

Date :

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CONTENTS

INTRODUCTION

Keratitis is an inflammation of the cornea caused by infectious organisms or non infectious agents. Microbial keratitis is potentially a vision threatening condition that can be caused by bacteria, viruses, fungi or parasites. Infectious keratitis is a significant public health problem. The reported incidence range from 11 per 1,00,000 person years in the United States to 799 per 1,00,000 person years in the developing nations like Nepal. In India the annual incidence is reported to be 11.3 per 10,000. Infectious keratitis requires prompt diagnosis and treatment to prevent blindness or even enucleation.

Few clinical signs distinguish infectious keratitis from corneal inflammation associated with trauma, hypersensitivity and immune mediated conditions. Diagnosis is assisted by the patient's history and ocular examination, focusing on the presence or absence of an epithelial defect and stromal inflammation. Microbiological tests are needed to establish aetiological agents and antimicrobial susceptibility.Presumptive treatment of the keratitis is often begun immediately after specimens are obtained for isolation.The regimen may be changed based on reports of culture and antimicrobial susceptibility test.

Given the rapid progression and virulent nature of many infectious agents, any corneal inflammation should be considered a threat to vision, requiring prompt evaluation and treatment.

Subsequent endophthalmitis (inflammatory process involving the ocular cavity and adjacent structures), leading to loss of vision or even loss of the eye is an ever present danger in such settings.

Cornea is a transparent avascular structure which consists of 5 layers.

1. Corneal epithelium with its basement membrane
2. Bowman's membrane
3. Substantia propria (stroma)
4. Descemet's membrane
5. Endothelium

Normal mechanisms which prevent corneal ulcerations include

* Eyelid – is a physical barrier providing protection against mechanical injuries.
* Smooth corneal surface with intact epithelium
* Tear film containing enzymes combined with the mechanical action of blinking eyelids, reduces the likelihood of microbial attachment

and survival on the corneal surface.

Generally microbial agents do not cause keratitis in immuno competent hosts or hosts without prior epithelial injury. There are exceptions however in which organisms such as *Neisseria gonorrhoea*, *Listeria monocytogens*, *shigella* and *corynebacterium* spp, may invade an intact epithelial surface.

Corneal Ulcer :

Is an inflammatory or more seriously infective condition of the cornea involving disruption of its epithelial layer with involvement of corneal stroma.

Predisposing risk factors associated with microbial keratitis usually involve disruption of the corneal epithelium such as wearing of contact lenses, trauma (Iatrogenic and traumatic), contaminated ocular medications, and altered structure of the corneal surface.

Contributing risk factors include diabetes mellitus, immunodeficiency, exposure keratoplasty (eg. Grave's exophthalmopathy, Bell's palsy). Surface alterations from or with dysfunctional tear states (eg. Sjogren's syndrome, neurotrophic cornea, chemical burn, Steven Johnson syndrome, medication related) and anatomical abnormalities (eg. Neoplasia, cicatrical pemphigoid and traumatic lid scarring)

Ocular trauma other than corneal surgery repeatedly account for 48% to 65% of all corneal ulcers in some developing countries. But such trauma was responsible for only 27% of corneal ulcer in U.S., whereas in India trauma accounts for 60% of the corneal ulceration.

Contact lenses are the most common risk factor for microbial keratitis diagnosed in the US. The annual incidence of contact lens associated keratitis is estimated at 0.04% for individual with daily wear soft lenses and 0.21% for individuals with extended wear lenses.

Several studies have reported that bacterial pathogens are responsible for most of the cases of microbial keratitis.

Most of the bacterial keratitis are caused by 5 major groups. *Staphylococcus* spp, *streptococcus* spp, (*streptococcus pneumoniae*, Group A through G. *Streptococci)* other Gram positive organisms (*Bacillus* and *Propionobacterium* spp) Gram negative organisms (eg *Pseudomonas, Hemophilus* and *Moroxella*) and the *Enterobacteriacae*, *(Proteus, Klebsiella, Enterobacter* and *Citrobacter)*

With the advent of refractive surgery, especially Laser Assisted Insitu Kerato Mileusis (LASIK), more unusual organisms such as *Nocardia* and *Mycobacterium* spp are also causing keratitis.

The apparent change in the causal organisms could be the result of numerous factors such as improved isolation techniques, increased use of topical corticosteroid (ie. Refractive and cataract surgery) increased population of immuno deficient patients and an expansion in the use of soft contact lenses, especially extended wear and cosmetic lenses.

Fungi are generally responsible for less than 10% of corneal infections in most clinical cases reported in the United States whereas in India,fungal keratitis accounts for more than 60% of the cases. Keratitis due to moulds occur more commonly in areas with a warmer and more humid environment.

The fungi are usually inoculated into the cornea by trauma involving plant or vegetable matter.

Topical cortico steroids for medical or surgical ocular conditions (LASIK) and the use of soft contact lenses as a bandage for post operative or damaged corneas may increase the likelihood of fungal keratitis.

The incidence of fungal keratitis varies according to geographic location and ranges from 2% in NewYork to 35% in Florida. *Fusarium* spp are the most common cause of fungal corneal infection in the Southern US whereas *candida* and *Aspergillus* spp are more common in the Northern States.In India *Fusarium* species are the most common organisms followed

by *Aspergillus* species.

Patients with fungal keratitis generally have fewer inflammatory signs and symptoms than patients with bacterial keratitis.

In 2006, the CDC began to receive reports of an increased incidence of contact lens associated Keratitis.

Major predisposing risk factors for keratitis resulting from *Candida* spp are prolonged epithelial ulceration, topical cortico steroid use, recent keratoplasty and current use of a bandage soft contact lens (ie. Recurrent erosion, persistent epithelial defect).

Fungal keratitis remains a diagnostic and therapeutic challenge. Difficulties are related to establishing a clinical diagnosis, isolating the causative agent in the laboratory and treating the keratitis effectively with topical antifungal agents.

Even if the diagnosis is made accurately, management remains a challenge because of the poor corneal penetration and limited commercial availability of antifungal agents

The small area of active infection and the need to avoid excessive corneal thinning by unnecessary scraping needs ocular akinesia and patient cooperation.

This may be accomplished through use of topical anaesthetics in patients old enough to cooperate, with general anaesthesia potentially needed in children.

Specimens are collected by using sterile surgical blades, blunt platinum spatulas or calcium alginate swab (often dipped in trypticase soybroth).

Materials from the scraping is transferred directly to glass slides and appropriate culture media. The slides should be clean to avoid artifacts and sterile to avoid contaminating the instrument. Multiple slides are desirable to permit Gram stain, calcoflour and KOH wet mount and acid fast stain.

If the patient had been treated before evaluation, and there is uncertainty regarding the diagnosis, it may be wise to consider stopping the medication for 12 to 24 hrs and then proceeding with culture. Antimicrobials should not be stopped in cases of severe or rapidly progressive destruction.

As a clinical routine for microbiologic evaluation of the patient with suspected keratitis, direct inoculation of material from corneal scrapings into blood, chocolate and Sabouraud's agar plates with 'C' Streaks provide the support for growth of majority of bacterial and fungal pathogens.

Liquid thioglycollate broth is then inoculated by transferring the

material from corneal scrapings from the spatula or surgical blade to a cotton tipped applicator or calcium alginate swab. The swab is then inserted into the bottom of the tube to enhance the growth of possible anaerobic pathogens.

Aerobic and anaerobic cultures of the corneal scraping should be incubated for 7 days before being reported as no growth. Mycobacterial and fungal cultures should be incubated for 4 to 6 weeks before being reported as no growth.

The results of corneal cultures should be interpreted with regard to the clinical situation, the adequacy of sampling and the possibility of contamination by organisms present on the skin, eyelids and conjunctiva.

Supportive evidence for a pathogenic role of species are growth on two or more media, heavy growth of the organism and a Gram stain directly smeared from the lesion containing organisms compatible with those isolated from culture.

Antibiotic sensitivity testing was performed by Kirby- Bauer disc diffusion technique, using 0.5 Mac Farland's turbidity as the standard inoculum's density on Mueller Hinton agar plates.

The recent increased incidence of fungal infections and the growing

number of newer antifungal agents have multiplied the demand and interest for invitro antifungal susceptibility testing.

WHO **Treatment Guidelines for the treatment of corneal ulcers:**

No fungal hyphae seen on smear	**Fungal hyphae seen on smear**
Cefazolin 5% and Gentamycin 1.4% drops hourly	Natamycin 5% drops hourly alone (no antibiotics)
Ciprofloxacin may be used instead of gentamycin. - if hourly drops is not possible - then a sub-conjunctival inj. can be considered.	Or Amphotericin B 0.15% drops hourly

Treatment frequency, duration and followup:

- Daily examination until the ulcer starts improving	- Examination every 2 days until the ulcer starts improving
- Then gradually reduce the frequency of drops and follow up over 2 weeks	- Then continue drops at least 3 hourly for at least 2 weeks after healing of the ulcer.

Refer to tertiary ophthalmic centre if:

Not improving after 3 days treatment	Not improving after 7 days treatment

Adjunctive therapy:

- Includes cycloplegics; analgesics; anti-glaucoma medication if indicated.
- Do not use any preparation containing steroids.

Investigate for diabetes mellitus as a possible risk factor for corneal ulceration

REVIEW OF LITERATURE

According to the National programme for control of blindness in 1992 Ministry of Health and Family Welfare, New Delhi, the number of blind people in the world is 45 million. Out of which 5.4 million blind people are in our country. corneal ulcer is a major cause of blindness through out the world. About 10% cases of blindness are due to corneal ulceration[68].

Bharathi MJ *et al* from South India in 2003 reported that microbial keratitis is a major cause of corneal opacity and loss of vision world wide[8].

Boucier T *et al* in 2003 from US has reported that the most common causative organisms are bacteria although fungi and protists are also pathogens [10].

M.J. Bharathi *et al* in 2003 from South india has reported that the epidemiology and etiology of bacterial keratitis is specific to the region. Screening patients for predisposing factors, treating the co-existing ocular diseases, and educating them about proper lens care and risk of infection may reduce the occurrence of bacterial keratitis[8].

Green M *et al* in 2008 from US has reported that several specific risk factors have been identified[34].

Cesar *et al* in 2008 from UK says that trauma is the leading predisposing factor[13].

Dr. Rajan K. Anand in 2010 from Bihar has reported that corneal ulcer is a common vision threatening condition among the rural population, next only to cataract. The annual incidence in India is reported to be 11.3 per 10,000[85].

Geetha Kumari *et al* from Kerala in 2011 has reported that the regional information of aetiological agent is very important as this will help us to have a high degree of clinical suspicion in starting the appropriate initial treatment before getting the microbiological confirmation[28].

This information will also help primary and secondary care ophthalmologists in initiating therapy as many of these centers lack adequate microbiology facilities.

Singh SK *et al* from Nepal in 2011 has reported that the incidence of corneal ulceration in Nepal is one of the highest reported in the world. The Bhaktapur Eye study revealed it to be 799 per 100,000 population per year. (Upadhyay et al, 2001) which is seven times higher than in South India (Gonzales et al, 1996) and seventy times greater that reported in the USA (Erie JC et al, 1993)[95].

B.H. Jeng *et al* in 2003 in US has reported that the highest rate of corneal ulceration was found in females (63%)[46,47].

Youhanna HW Ibrahim *et al* in 2009 from UK has reported predominance of corneal ulcer in female (54%)[119].

Sadia Sethi *et al* in 2010 from Peshawar (India) has reported that the incidence of microbial keratitis was high in males (67%)[89].

M.Srinivasan *et al* in 1997 from Madurai has reported increased incidence of corneal ulceration in males (65%)[97].

B.H. Jeng *et al* in 2003 from UK has reported that risk factors for corneal ulceration included contact lens use (55%), ocular surface disease (16.6%), trauma (11.9%), and bullous keratopathy (1.3%)[46].

Youhanna HW Ibrahim *et al* in 2009 from UK has reported that the contact lens wear was the main predisposing factor in (31%)[119].

M.Srinivasan *et al* in 1997 from Madurai has reported that corneal injury (65.4%) was the major predisposing factor in the aetiology of corneal ulcer[97].

Sadia Sethi *et al* from Peshawar in 2010 has reported that ocular trauma was the most common cause found in 39% of patients[89].

Reema nath *et al* from Upper Assam in 2011 has reported that injury

with vegetative matter is the most common risk factor[86].

Youhanna *et al* in 2009 from UK has reported that among the bacterial isolates, Staphylococcus aureus is the predominant organism (71.1%) [119.]

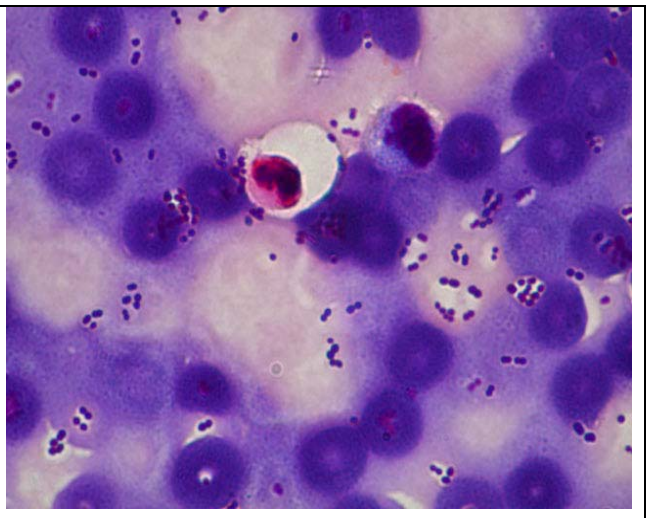
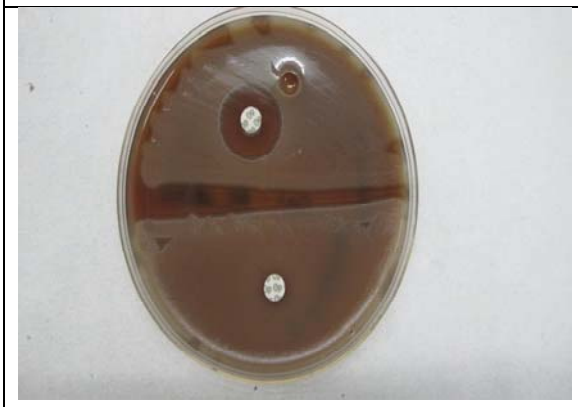
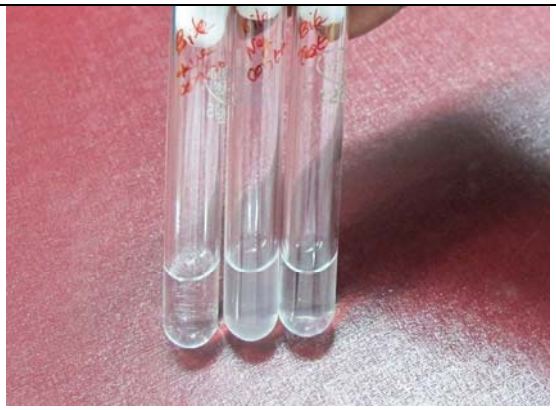
Sadia Sethi *et al* in 2010 from Peshawar has reported that *Pseudomonas* spp was the most common organism cultured in 50% of cases[89].

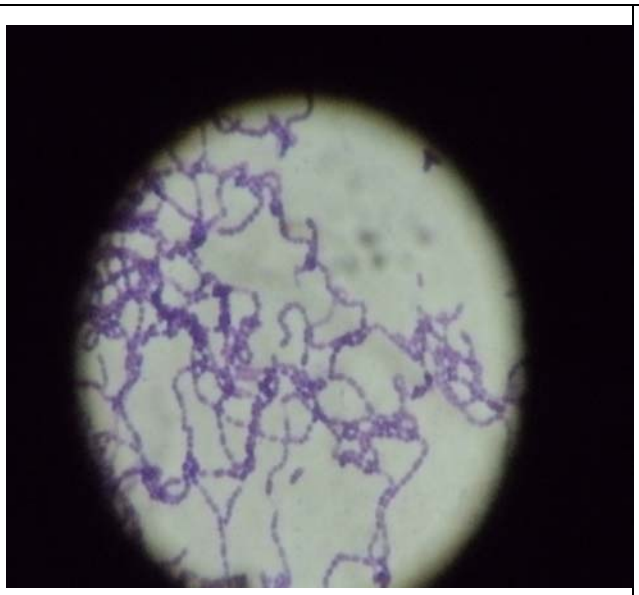
M. Jayahar Bharathi *et al* from South India Tamil Nadu in 2007 has reported that *S.Pneumoniae* (63%) was the predominant organism isolated from corneal ulcers[43].

Feilmeir *et al* in 2010 from Nepal has said that fungal organisms are the most common cause of infectious keratitis in patient population. *Aspergillus* (35%) among fungus and *S.Pneumoniae* among bacteria were the most common organisms responsible for keratitis[24].

M.Srinivasan *et al* from Madurai in 1997 has reported that *S.pneumoniae* (44.3%) was the predominant organism followed by *Pseudomonas* spp and the most common fungal pathogen isolated was *Fusarium* spp(47.1%) followed by *Aspergillus* spp. (16.1%)[97]

Jayahar Bharathi *et al* in 2007 from South India has reported that the incidence of fungal keratitis (66%) was more with agricultural workers and

	
Streptococcus Pneumoniae with alpha hemolysis	**Streptococcus Pneumoniae (Microscopic)**
	
Streptococcus Pneumoniae (Optochin sensitive) & Streptococcus Viridans (Optochin resistant)	**Streptococcus Pneumoniae (Bile solubility)**

	
Streptococcus Viridans (Microscopic)	**Nocardia (Chalky white colonies in BAP)**
	
Nocardia (Microscopic)	**Pseudomonas (Antibiogram)**

where as the bacterial keratitis (57%) was more common in non agricultural workers [43].

Noda AL yousuf *et al* in 2009 from Bahrain has reported that contact lens wear was the major risk factor for microbial keratitis in Bahrain. *Pseudomonas aeruginosa* was the most common bacteria isolated, sleeping with contact lens is the major risk factor among contact lens wearer[70].

Gogi *et al* in 1983 has reported that the most important cause of corneal ulceration was due to indiscriminate use of corticosteroids (or) due to lowering of host resistance as a result of acute (or) chronic ailments (or) systemic steroid therapy[31].

Ferrec C *et al* in 2011 from US reported that LASIK treatment is a predisposing factor for bacterial keratitis even years after surgery[26].

Prashant Garg *et al* from Vadavalli in 2010 reported that the incidence of this complication is estimated to be 1 in 5000 procedures[83].

Jorma B. Mueller *et al* in 2008 reported that prolonged exposure to UV light (or) brief exposure to intense UV light flashes can produce photokeratitis of non infectious origin[49].

According to Jagadish Chander *et al* from Chandigarh in 2008, the prevalent organisms involved in microbial keratitis were *Aspergillus* spp. (41.18%), *Fusarium* species (27%), *Candida* species (8.82%), *Curvularia*

(5.88%) and *Bipolaris* species (5.88%)[42].

Samar K Basak *et al* from West Benghal in 2005 reported that fungal ulcers are more common than bacterial ulcers. *Aspergillus* and *Staphylococcus aureus* were the most common fungus and bacteria respectively[90].

MJ. Bharathi *et al* in 2003 from South India has reported that a high index of suspicion of *Nocardia* infection should exist in patients with history of trauma to the eye by soil (or) sand[9].

Usha Arora *et al* in 2009 from Amristar has said that *Aspergillus* spp was the most common isolate followed by *Fusarium*, *Penicillium* and *Curvularia*[108].

Lisa J. Keay *et al* in 2011 from US has reported that trauma, contact lens wear and ocular surface disease predispose patients to developing fungal keratitis[58].

Laila Aktar *et al* in 2009 from Bangladesh has reported that *Pseudomonas* spp (24%), *S.Pneumoniae* (17%), *Aspergillus* spp (13%), *Fusarium* spp (7 %) and *Curvularia* spp (6%) were found as pathogens causing suppurative corneal ulcer[55].

Kursiah M.R.*et al* from Malaysia in 2008 reported that most of the contact lens induced corneal ulcer were caused by *Pseudomonas aeruginosa* and this finding will help in determining the empirical treatment to be initiated[54].

Philip Thomas from Trichy in 2002 has reported that the fungal infection of the cornea continues to be an important cause of ocular morbidity, particularly in the agricultural communities of the developing world. A proper understanding of agent and host factors involved in these infections will improve the outcome of this condition[103].

In 2008, in an eye camp conducted at Perambalur by a private hospital,66 patients underwent surgery for cataract.Postoperatively,the patients developed pain and irritation of eyes followed by loss of vision.The reason for loss of vision was attributed to the use of contaminated fluid during surgery.(The Times of India,Madurai/Trichy Wednesday,September 21,2011).

Pathogenesis :

Adherence of microbes to cornea

↓

Invasion into corneal stroma

↓

Inflammation and neovascularisation

↓

Interruption of the host immune response

↓

Stromal degradative process

Penetration of exogenous organisms into the corneal epithelium typically requires a defect in the surface of squamous epithelial layer.

By virtue of specialized enzymes and virulence factors a few bacteria such as *N. gonorrhoea, N. meningitidis, C. diphtheriae, Shigella* and *Listeria* may directly penetrate the corneal epithelium.

Reichert R *et al* in 1984 reported that the adherence of *S. aureus, S.pneumoniae* and *Pseudomonas* to ulcerated corneal epithelium is significantly higher than other bacteria and may account in part for their frequent isolation[87].

Haepelman AIM *et al* in 1992 reported that receptor recognition is only the first step in the pathogenesis of infection directed by microbial adhesion molecules[39].

Hyndiuk RA *et al* (in 1981) reported that in addition to adhesions, the adherence of *P. aeruginosa* and *N.gonorrhoea* to adhere to susceptible cells producing slime aggregates that are resistant to phagocytosis[40].

Koch JM *et al* in 1990 reported that similar coatings may form on contact lenses to facilitate adherence of bacteria to the lens material[52].

Clinical manifestations :

Patients generally present with complaints of pain, redness, reflex watering, photophobia and diminished vision.

On examination there may be conjunctival chemosis, congestion, purulent discharge, hypopyon and stromal infiltration.

Feilmeier, Michael R *et al* from Nepal in 2010 has reported that smear microscopy is reliable in determining the etiology of the corneal infection and can be used to help guide initial therapy in this setting[24].

Wilhelmus KR et al reported that (in 1994) laboratory diagnosis of ocular infection by culture is the gold standard for clinical management. Standard laboratory procedures can usually identify most of the organisms

by stain or culture[115].

If the patient had been treated before evaluation and there is uncertainity regarding the diagnosis it may be wise to consider stopping the medication for 12 to 24 hrs and then proceeding with culture.

Antimicrobials should not be stopped in cases of severe or rapidly progressive ulceration.

Agarwal V *et al* in 1994 reported that corneal scraping are collected under strict aseptic precautions by an ophthalmologist, using sterile No.15 Bard Parker blade after instillation of local anaesthetics like 2% lignocaine hydrochloride from the leading edge of the ulcer[1].

Gram Staining :

Noopur Gupta *et al* in 2008 reported that smears prepared by corneal scraping and Gram staining done to observe the bacteria and yeast cells[71].

Bharathi et al in 2006 reported 100% sensitivity of Gram stain procedure in the diagnosis[7].

Gomez *et al* in 1988 and **Groden *et al*** in 1990 reported that the acridine orange stain accurately predicts culture results in 71%, 84% of cases compared to 62-79% for the Gram stain[32,35].

Vajpayee *et al* in 1993 reported that 10% KOH mount demonstrate fungal elements in 94.3% of total culture positive cases of keratomycoses[111].

Chowdhry *et al* in 2005 reported that direct microscopic examination of KOH mount is a rapid, reliable and inexpensive diagnostic modality, which would facilitate the institution of early antifungal therapy before culture reports become available thus providing to be sight saving[14].

In 1998, **Silverberg *et al*** reported that 10% KOH mount positive in 100% total culture proven cases[94].

Usha Gopinathan *et al* in 2008 from Hyderabad stated that simple KOH preparation of corneal scraping alone is highly beneficial in confirming the diagnosis[109].

Lactophenol cotton mount :

Thomas *et al* in 1991 documented the correlation of macroscopic morphology with microscopic findings in LPCB mount[104].

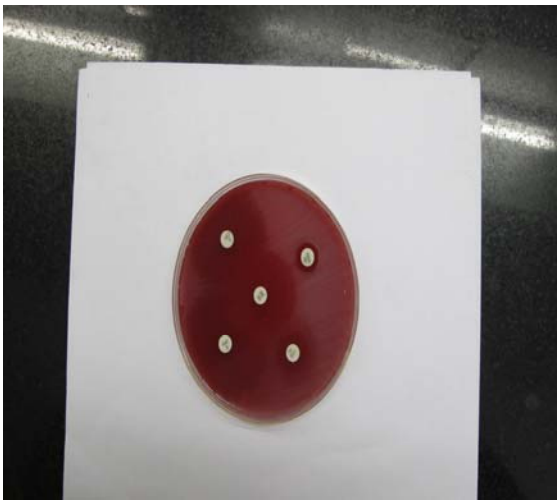
Culture :

Wihelmus *et al* in 1994 reported that the culture media recommended for evaluation of suspected microbial keratitis have the potential to support the growth of the principal bacteria and fungi responsible for keratitis[115].

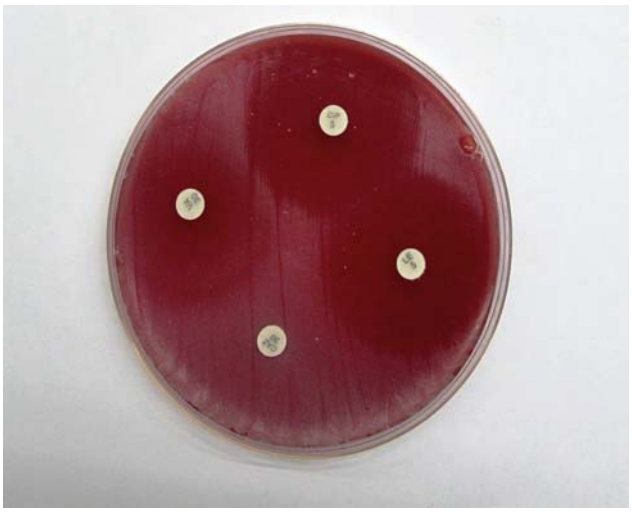
'O'Brien *et al* in 1994 said that the SDA agar should not contain cycloheximide which may inhibit the saprophytic fungi commonly responsible for ocular infection[72].

Commonly used Culture media

Medium	Organism that can be cultured
Standard media	
Blood agar	Aerobic, Facultative anaerobic bacteria and fungi
Chocolate agar	Aerobic, Facultative anaerobic bacteria , fungi + *Neisseria* and *Hemophilus*
SDA with Gentamycin	Fungi
Thioglycollate broth	Aerobic and Anaerobic bacteria
Additional media	
BHI broth	Fungi
LJ Agar slant, middle brook agar slant	*Mycobacterium*
Schaedler's agar, Brucella agar	Anaerobic bacteria
Thayer martin agar	*Neisseria*
Non nutrient agar with E.coli overlay	*Acanthamoeba*

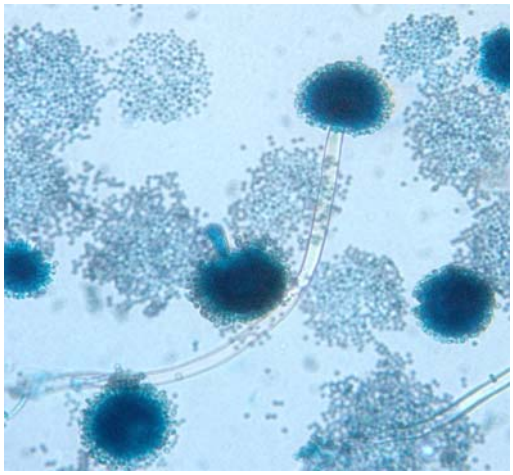


S. viridans (antobiogram)



Nocardia (Antibiogram)

Aspergillus flavus (Macroscopic)



Aspergillus flavus (Microscopic)

Anti microbial susceptibility testing :

According to CLSI (Clinical & Laboratory Standard Institute) standard disc diffusion or micro dilution are the preferred lab methods for anti microbial susceptibility testing of ocular bacterial isolates.

Antibiotic sensitivity testing was performed by the Kirby-Bauer disc diffusion technique, using 0.5 Mac Farland's turbidity as the standard inoculum's density on Mueller Hinton agar plates.

Antifungal susceptibility testing :

The recent increased incidence of fungal infections and the growing number of new antifungal agents have multiplied the demand and interest for invitro antifungal susceptibility testing.

The CLSI sub committee on antifungal sensitivity has developed both micro dilution and disc diffusion method for testing susceptibility of filamentous fungi.

Recent methods :

Polymerase chain reaction amplification can be used to detect the presence of as low as 10 organisms per 100 ml volume of clinical specimen. Corneal scrapings are processed for DNA extraction which is amplified by fungal specific primer of internal transcribed spacer region. (ITSI). The

products are sequenced and analysed by single standard confirmation polymorphism for species identification.

Motoki Hahashi *et al* in 2010 have stated that the real time PCR can accurately and simultaneously detect bacterial and fungal pathogens in a speedy fashion[66].

Itahashi M *et al* has reported that the real time PCR can simultaneously detect and quantitate bacterial and fungal pathogens in patients with corneal ulcer. Real time PCR can be a test diagnostic tool and may be useful as an adjunct to identify potential pathogens[41].

ELMA KIM *et al* in 2008 has stated that yield and concordance with culture are higher for fungal than bacterial ulcer[20,21].

Ferrer *et al* in 2002 highlighted the benefit of time factor in diagnosing fungal corneal ulcer. PCR assay produced results in 8 hrs, culture confirmation took almost 10 days[26].

Sujith venayil *et al* in 2009 has reported that although PCR has several advantages due to its rapidly and wide spread applicability to bacteria, fungi and viruses the technique has various reported complexities and drawbacks as evidenced from their study also some of the limitations are logistic and some are technical[100].

Among them is the difficulty in optimization especially in case of fungi, apart from the difficulty in differentiating between active and latent infections, viable and non viable cells and high chance of false positivity can be caused by lab contaminants from reagents, intra sample contamination, and processing of positive control specimens.

Elma Kim et al in 2008 in a study from Madurai has stated that PCR detects microbial DNA in the majority of the bacterial and fungal corneal ulcers and identifies potentially pathogenic organisms in a high proportion of culture negative cases. Yield and concordance with culture are higher for fungal than bacterial ulcers[20,21].

Practical use of the technique is limited by artifactual amplification of non pathogenic organisms, PCR may be used as an adjunct to culture to identify potential pathogens in microbial keratitis

Management of bacterial keratitis :

Topical administration is the method of administration of choice. Because it provides a rapid high level of drug in the cornea and anterior chamber.

Baccum J *et al* in 1983 reported that subconjunctival injection is associated with increased pain and inflammation, patient apprehension and

risk of globe perforation while failing to provide enhanced corneal levels of antibiotics compared with topical drops[4].

Davis SD *et al* reported that oral or parenteral administration establishes a relatively low level of antibiotic in the cornea and does not appear to contribute to the effect of topically applied drug[17].

Systemic antibiotics are advised only when keratitis is complicated by scleritis or there is a risk of perforation or endophthalmitis.

Significantly higher corneal level of drugs can be established with more frequent application of drops.

'O' Brien TP *et al* in 1995 and **Panda A *et al*** in 1991 reported that initial regimens of fluroquinolone or aminoglycoside combined with a cephalosporins is effective in approximately 95% of cases of bacterial keratitis[74,77].

All fluroquinolones demonstrate excellent activity against Gram negative organisms with good to excellent activity against Gram positive organisms but variable activity against anaerobes and S. Pneumoniae.

Ciprofloxacin remains the fluroquinolone of choice for pseudomonas.

Amikacin is a semi synthetic aminoglycoside that is useful in the treatment of infection due to gram negative infection resistant to gentamycin

and tobramycin.

Fiscella RG et al in 1995 reported that to minimize the development of resistance, empirical therapy is not encouraged. *Nocardia* infection is also responsive to treatment with sulfonamides. A combination of Trimethoprim and sulfamethoxazole to be administered both topically and systemically[27].

Management of fungal keratitis :

Stephen keye *et al* in 2010 from UK has said that topical application of an antimicrobial to the cornea may achieve a very different tissue concentration and bioavailability than in the serum[98.]

Sonali. S. Tuli from US in 2011 has reported that topical Natamycin is the most commonly used medication for filamentous fungi while Amphotericin B is the most commonly used for yeast. Voriconazole is rapidly becoming the drug of choice for all fungal keratitis, because of its wide spectrum of coverage and increased penetration into the cornea[96].

Thomas PA *et al* in 2003 from India says that Natamycin (5%) (or) Amphotericin B (.15%) remain the drug of choice for superficial keratitis[105].

Therese K.L. *et al* in 2006 from Malaysia has reported that among filamentous fungi, *Asp.niger* followed by *Asp.terreus* exhibited higher percentage of resistance to Amphotericin B[102].

Usha Arora *et al* in 2006 from Amristar has reported that 81% of *Aspergillus* species were resistant to Flucanazole[107].

Pankaj K Agarwal *et al* in 2001 from Calcutta has reported that Itraconazole is effective in treating mycotic corneal ulcers[78].

Usha Gopinathan *et al* in 2009 from South India has reported that a significantly large no of patients with fungal Keratitis required surgical (50.8%) intervention compared to bacterial Keratitis thus indicating a poor response to treatment in fungal keratitis[110].

AIMS AND OBJECTIVES

- To find out the etiological agents causing corneal ulcer.
- To identify the predisposing factors causing corneal ulcers
- To find out the role of cofactors like age, sex, occupation predisposing to corneal ulcers.
- To find out the anti microbial sensitivity pattern of bacterial and fungal pathogens isolated.

MATERIALS AND METHODS

The study group comprised of 120 patients attending the cornea clinic at Department of Ophthalmology, Govt. Rajaji Hospital, Madurai (tertiary care hospital) and Aravind Eye Hospital(a private sector hospital dedicated to Ophthalmology), Madurai during the period from December 2010 to July 2011 The Institutional ethical committee clearance was obtained for study .

INCLUSION CRITERIA:

Patients having proven corneal ulcer on clinical examination.

Both outpatient and inpatient were included in the study.

Postoperative patients of ocular surgery with suspicion of impending corneal ulcer.

COLLECTION OF SPECIMENS:

Written consent from the participants (or) their guardians included in the study was obtained after providing full explanation of the current study in their local language. All the data collected were kept confidential.

Specimens were collected from patients with corneal ulcer and follow-up patients with corneal ulcer. Informed consent was obtained from

the patients and data were collected as per proforma. Corneal scrapings were collected for investigations by the Ophthalmologist.

6. Patient was made to lie down comfortably on a couch.
7. The affected eye was cleansed with sterile saline using sterile swabs.
8. Sterile 2% Xylocaine was applied to the eye taking care not to apply too much of it as it may inhibit the growth of the organism.
9. Care was taken to see that the eyelids did not contaminate the specimens. Eye speculum was used whenever necessary.
10. Patients were given relevant instructions regarding position and restriction of eyeball movement during the scraping procedure.
11. No.15 and Bard Parker blades were used to scrap the ulcer. A new sterile blade was used for each patient.
12. The corneal scraping was inoculated in a C. Streak pattern on culture media (Blood agar, chocolate agar, potato dextrose agar, sabouraud's agar).
13. Direct Gram's staining and 10% KOH wet mount were made on the direct scraping.
14. Blood agar and chocolate agar plates were incubated at 37^0c in the presence of 5% Co_2 for 2-7 days.

15. Sabouraud's dextrose agar slant and potato dextrose agar slant were incubated at 25^0C aerobically.
16. The culture plates and slants were looked for the growth of organisms.
17. If bacterial growth was observed, staining (Gram's and modified acid fast) was performed.
18. Biochemical tests were done to identify the pathogen.
19. Antibiotic sensitivity pattern was performed to identify the sensitivity pattern of pathogens to the antibiotics.

If the fungal growth was observed, lactophenol cotton blue staining was performed and fungus was identified based on the spore morphology.

SPECIMEN PROCESSING:

The following tests were performed on the specimens that were collected.

Gram staining

1. Thin smear of the specimen was prepared on a clean sterile glass slide.

2. Then the smear was fixed by heating over a bunsen burner flame.

3. The smear was flooded with 1% gentian violet for 1 minute & washed with distilled water.

4. The smear was flooded with gram's iodine for 1 minute and washed

with distilled water.

5. and decolorized with acetone, washed with distilled water and counter stained with dilute carbol fuschin for 30 seconds.

Modified acid fast staining

1. Thin smear of the specimen was prepared and dried in the air.

2. The smear was fixed by heating over a Bunsen burner flame.

3. The smear was flooded with strong carbol fuschin stain for 5 minutes.

4. Washed with distilled water and flooded with 1% sulphuric acid for 3 minutes.

5. Washed with distilled water and counter stained with 3%methylene blue for 3 minutes.

6. Washed with distilled water, dried, and examined under oil immersion microscope.

KOH wet mount

- A clean glass slide was taken.
- The specimen was placed in the centre of the slide.
- A drop of 10% KOH was added and a coverslip was placed over that.

- and observed under microscope.

Lactophenol cotton blue staining

- With the help of a sterile teasing needle a small fragment of the colony to be identified was taken.
- A drop of lactophenol cotton blue stain was placed in the centre of the slide.
- By using teasing needles, the growth was spread over the slide and the coverslip was placed without trapping any air bubbles.
- Under low power and high power objective, the morphology of hyphae, conidia were observed and was correlated with macroscopic features.

Slide culture method:

This was done to see the morphology of structures of fungi such as spores, conidiophores and hyphae.

1. A round piece of filter paper was placed on the bottom of Sterile Petri dish. A pair of thin glass rods was placed over the filter paper.
2. A 3 inch x 1 inch glass microscopic slide was placed over the glass rods.
3. 1 x 1cm square block of sabouraud's dextrose agar was cut from a Petri

dish with the help of sterile scalpel and the agar block was transformed to the microscope slide.

4. The fungal colony was inoculated into four sides of the agar block by using sterile needle.
5. The agar block was covered with sterile coverslip in the Petri dish.
6. Moistened filter paper was placed within the Petri dish.
7. The Petri dish was incubated at room temperature and examined for growth periodically.
8. When a growth appeared visually, the coverslip was removed from the surface of the agar block with forceps.
9. The coverslip was placed on a drop of lactophenol cotton blue stain on a glass slide.
10. Like wise, the agar block was removed and the fungal growth adhering to the surface of the microscopic slide was stained with lactophenol cotton blue and new coverslip was placed over that.
11. The shape and arrangement of conidia were observed microscopically.

Microbial culture were considered significant,

a) If growth of same organism observed in more than one culture slope (or)

plate.

b) If there was confluent growth at the site of inoculation in solid media.

c) Growth was consistent with microscopic findings (KOH mount, Gram stain and modified acid fast stain).

d) If the same organism was grown from repeated scraping from the patients.

Interpretation of Bacterial culture:

Bacterial culture plates were observed for growth at 24 hrs and 48 hrs. Any growth seen outside the 'C' streak was considered as contaminant. Bacterial isolates were identified by means of Gram's staining, motility and biochemical reactions by standard microbiological techniques as recommended by Clinical and Laboratory Standard Institute (CLSI).

Interpretation of Fungal culture:

Inoculated SDA slants were incubated at 30^0C for minimum of 4 weeks before discarding as negative. These slants were inspected daily during the first week and twice weekly during the next three weeks. Growth on two slants or growth on one medium with presence of hyphal elements in 10% KOH preparations was regarded as significant fungal growth. Identification of filamentous fungi was done by preparing Lacto Phenol Cotton Blue

mount and studying the morphology of hyphal and conidial arrangement.

SENSITIVITY TESTING OF ISOLATES

ANTIBACTERIAL SENSITIVITY TEST

Bacterial isolates were subjected to antibiotic sensitivity testing by the Kirby-Bauer's Disc Diffusion technique on Mueller Hinton agar plates as recommended by CLSI. Peptone water culture of the bacterial isolates corresponding to 0.5 McFarland's turbidity was used as inoculum.

The surface of Mueller-Hinton agar plate (after ensuring drying) was evenly swabbed in three different directions with a sterile cotton swab dipped into the inoculum Maximum six antibiotic discs were used for each 9 cm diameter plate. These plates were incubated at 37^0C for 16-18 hours in Ambient air. The diameters of zones of inhibition were interpreted according to CLSI standards for each organism. Media and discs were tested for quality control using standard strains.

The antibiotic discs used for bacterial isolates were: Gatifloxacin, Tobramycin, Ceftazidime, Vancomycin and Cotrimoxazole.

ANTI FUNGAL SUSCEPTIBILITY TESTS

The antifungal susceptibility testing was done by three methods.

Disc diffusion method

Broth microdilution method

Agar dilution method

The Clinical and Laboratory Standards Institute (CLSI) subcommittee on Antifungal Susceptibility Tests has developed a reproducible procedure for antifungal susceptibility testing of filamentous fungi by a broth microdilution. Recently, an agar diffusion method has been developed for testing filamentous fungi.

INVITRO SUSCEPTIBILITY TESTING:

The Clinical Laboratory Standard Institute (CLSI) which describes the standard parameters for testing MIC (Minimum Inhibitory Concentration) of established agents against filamentous fungi.

Antifungal susceptibility testing is receiving attention with the advent of newer anti fungal drugs. However susceptibility testing of filamentous fungi is not as advised as susceptibility testing. In vitro susceptibility tests should provide a reliable measure of relative activity of the antifungal agent, correlate with in vivo activity and predict the likely outcome of the therapy, provide a means with which to monitor the development of resistance and predict the therapeutic potentials of newer drugs.

Invitro susceptibility testing of fungi is influenced by a number of technical variables such as inoculums size and preparation, medium composition and pH, duration and temperature of incubation and MIC end point determination. In addition there are problems unique to fungi like their slow growth rates and the ability of some of them to grow either as yeasts with blastoconidia or as moulds with variety of conidia depending on pH, temperature and medium composition.

DISK DIFFUSION METHOD:

1. Inoculum preparation:

The fungal colony to be tested was grown in Potato dextrose agar slants at 35c to induce the conidium and sporangiospore formation. After 7 to 10 days of incubation with well grown spores, the culture was taken for testing.

This method dilution method was performed on Nutrient agar or Muller Hinton agar plates supplemented with 2% glucose.

The plate was allowed to dry for 10 minutes. Using a pair of flame sterilized forceps the antifungal disks were applied onto the surface of the inoculated plate. The plates were incubated at 35c for 48 hours. The plates were read at 24 hrs and 48 hrs.

The following commercial Hi-Media antifungal disks were used.

Amphotericin B 100units Itraconazole 10ug

Fluconazole 10 ug Nystatin 100 units

The following standard strains were tested each time to ensure quality control. Aspergillus flavus ATCC 204304

Aspergillus fumigatus ATCC 204305

2. Interpretation:

Zone diameters were measured at the point where there was prominent reduction of growth. The results were compared with broth microdilution method for respective fungal isolates.

AGAR DILUTION METHOD:

Procedure & Interpretation:

1) 1.8 ml of molten Nutrient agar poured into sterile test tubes and allowed to cool to $50^{o}C$.
2) 0.2 ml of drug dilutions from stock solution added in descending concentration to NA slope.
3) 100ul of standardised inoculums added to all tubes except sterility control tube.
4) Tubes incubated at $30^{0}C$ for 2 days.
5) Visualised macroscopically for growth.

6) Lowest concentration of the drug which permitted no macroscopically visible growth after 2-3 days is taken as MIC.

BROTH MICRODILUTION METHOD

1. Growth Medium Preparation:

1. The completely synthetic medium Rosewell Park Memorial Institute – 1640 (RPMI-1640) supplemented with 0.3g of L-glutamate per liter without sodium bicarbonate was used as a growth medium in antifungal susceptibility testing. The medium should be buffered at the pH of 7.0 - 7.2 at 35^{o}C.
2. The buffer used was MOPS (3-N-morpholinopropane sulfonic acid) with optimal concentration of 0.165 mol/L with pH of 7.0.
3. RPMI 1640 was dissolved in MOPS. The final solution was sterilised by filtration through membrane filter and stored at 4^{o}C.
4. The same medium was used for the preparation of the drug dilutions.

2. Drug Dilution Preparation:

1. The drug dilutions were prepared following the additive two fold drug dilution scheme described in the NCCLS M38-A method.
2. Stock drug solutions were first diluted to 100x the final concentration in 100% dimethyl sulfoxide (DMSO) and further diluted 1:50 in 2x

medium to obtain the 2x drug concentration. The final drug concentration was 0.125 to 32 for Amphotericin B and Itraconazole.

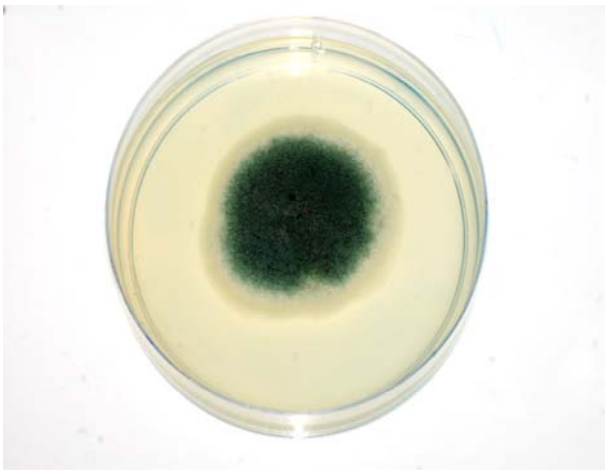
3. These volumes were adjusted according to the total number of tests required. Because there will be 1:2 dilution of the drug when combined with the inoculum, working antifungal solutions were 2 fold more concentrated than the final concentration.

3. Inoculation in RPMI – 1640 Medium:

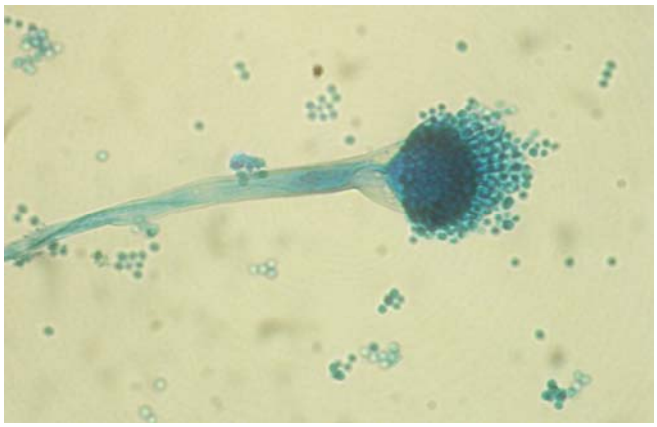
1. The inoculation was done in sterile 96 – well microlitre plate with flat bottom.
2. Each well was inoculated with 100 ul of the conidial suspension.
3. 100ul of the diluted drugs were added correspondingly to each well.
4. The growth control well was inoculated only with the 200 ul of diluted conidial suspension with the growth medium without any antifungal agents.
5. The sterility control well was inoculated with 200 ul of the growth medium alone without any conidium.
6. All microtitre plates were incubated at $35^{o}C$ for 48 hours without agitation and evaluation was done after four days of incubation.

4. Reading MIC

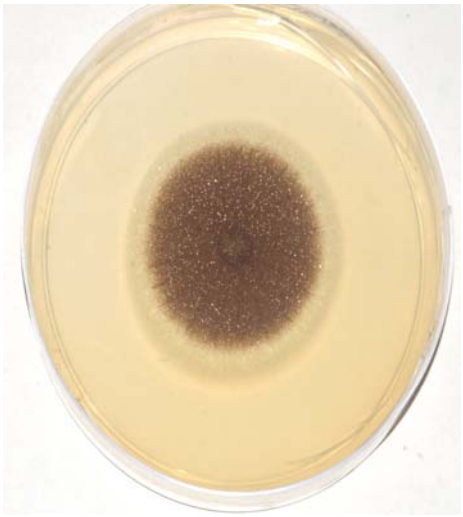
1. The test was read when the growth control shows adequate growth, which is typically 24-48 hours for most moulds, but it could be up to 96 hours.
2. Read MICs the first day that the growths controls showed the visible growth and then 24 hours later.
3. Scores were given as follows, (1) 0 – optically clear (2) 1 + = slightly hazy (3) 2± prominent reduction in turbidity compared with that of the drug-free growth control.
4. 3+ = slight reduction in turbidity compared with that of the drug-free growth control.
5. 4+ = no reduction in turbidity compared with that of the drug-free growth control.



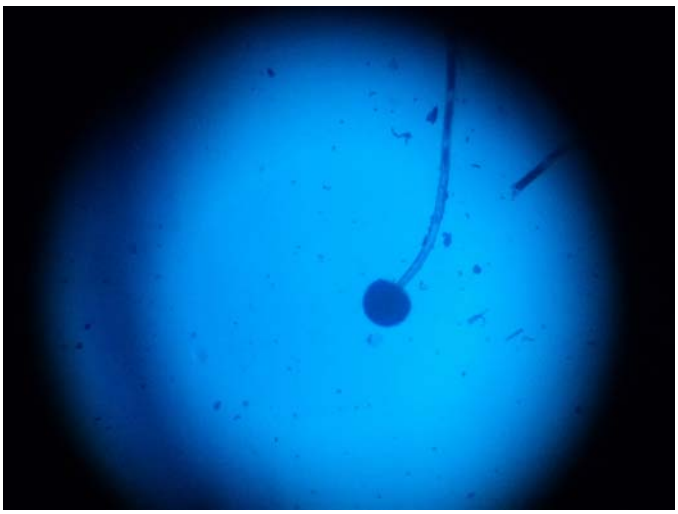
Aspergillus fumigatus (Macroscopic)



Aspergillus fumigatus (Microscopic)



Aspergillus niger (Macroscopic)



Aspergillus niger (Microscopic)

RESULT

A total of 120 patients with infectious corneal ulcer were selected for study. This study involves males and females of all age group. 75 cases were culture positive.

TABLE 1

CULTURE POSITIVITY IN THE CORNEAL SCRAPING SAMPLES

N = 120

Total No. of samples collected	No. of culture positive samples	% of culture positivity
120	75	62.85%

TABLE 2

SMEAR POSITIVITY AMONG CORNEAL ULCER ISOLATES

Gender	Total no of specimens	10% KOH positivity	Gram stain positivity
Male	77	46	45
Female	43	25	24

Sensitivity 96% Specificity 96%

FIGURE - 1

CULTURE POSITIVITY IN THE CORNEAL SCRAPING SAMPLES

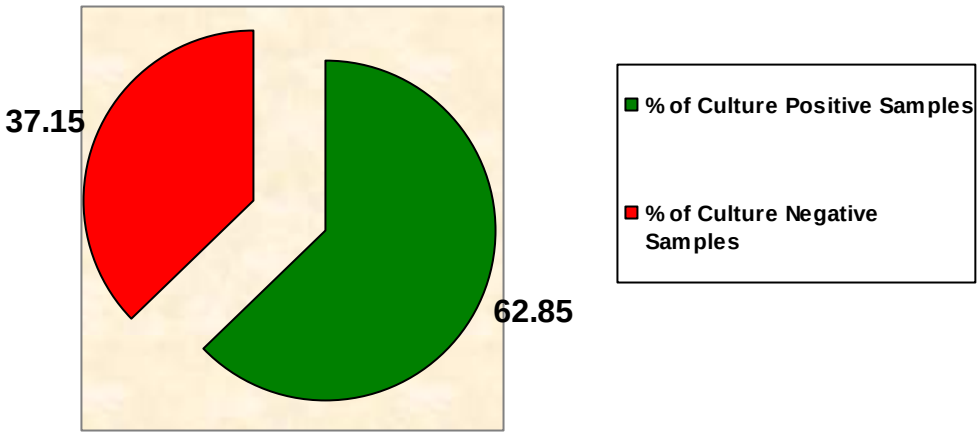
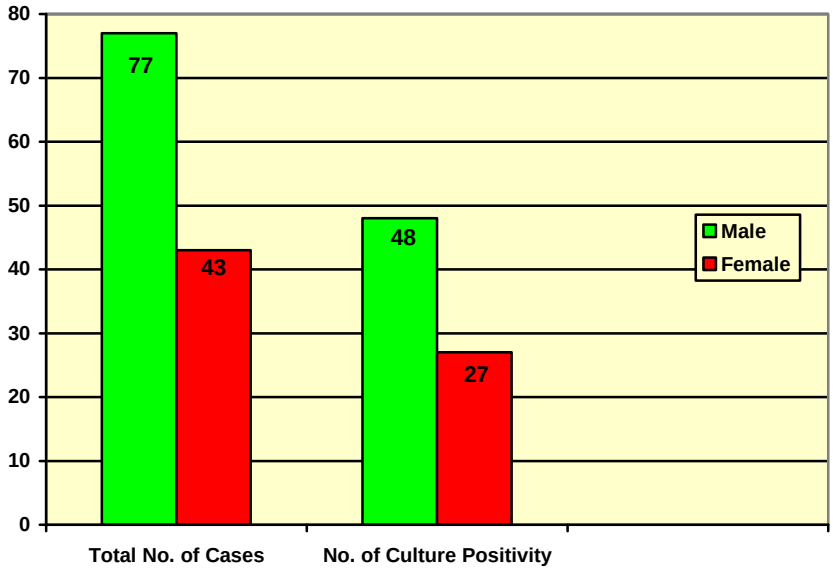


FIGURE - 2

GENDER DISTRIBUTION OF INFECTIOUS CORNEAL ULCER



The cases were analysed under the following parameters. Out of 120 cases 77 patients were male and 43 patients were female. (50/120) 41% cases were found to be in the age group between 30-60 years and 58.3 % (70/120) of cases were in the age group of > 60 years.

TABLE – 3

AGE DISTRIBUTION OF INFECTIOUS CORNEAL ULCER

N = 120

Age	Total No. of Cases	Male	Female	Percentage of Cases
0 – 30 years	50	40	10	41%
30 – 60 years	70	45	25	58.3%
Total	**120**	**85**	**35**	**100 %**

Considering the sex distribution, 48 (64.38%) males and 27 (35.6 %) female patients showed positive culture. A high prevalence of keratitis was seen among males contributing to 64.38% of cases.

Table 2 and 3 shows the age and sex distribution of the patients along with the positive culture for bacteria and fungi. This study shows that the maximum incidence of keratitis was seen in 3rd to 5th decade.

FIGURE – 3

AGE DISTRIBUTION OF INFECTIOUS CORNEAL ULCER

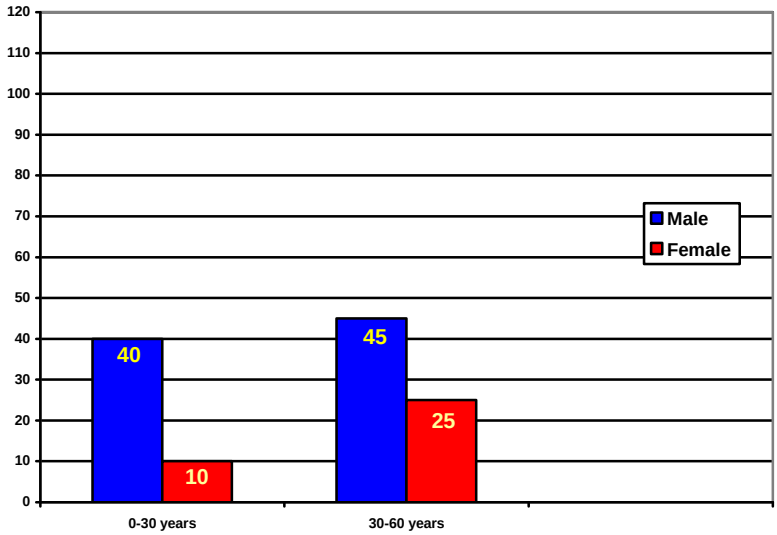
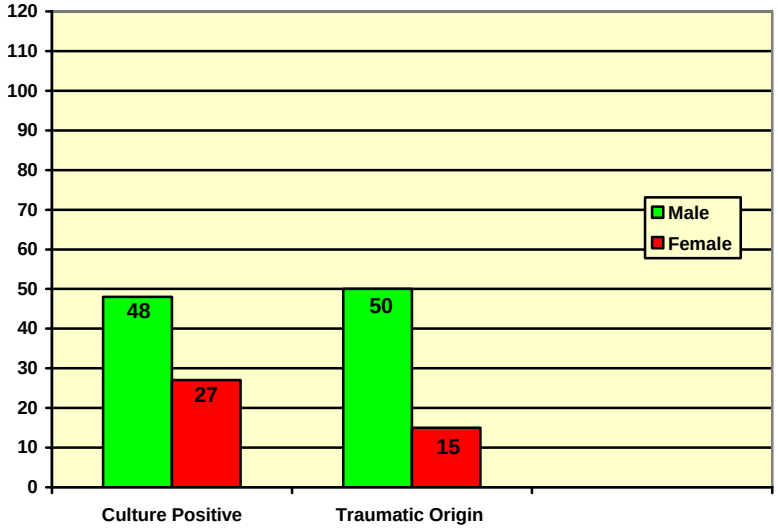


FIGURE – 4

DISTRIBUTION OF PREDISPOSING FACTORS CAUSING CORNEAL ULCER



The distribution of cases among rural and urban areas, showed increased prevalence of keratitis in rural population accounting for 70%.

TABLE – 4

DISTRIBUTION OF CASES AMONG RURAL AND URBAN AREAS

Total	Rural	Urban
120	84	36
Parentage %	70 %	30 %

Numerous predisposing factors have been implicated, trauma alone contributed to 54.16% of the cases in the development of keratitis.

TABLE – 5

DISTRIBUTION OF CORNEAL ULCER AMONG TRAUMATIC CASES N = 120

Nature of Trauma	**Male**	**Female**	**Total**	**%**
Vegetative matter	18	8	26	40%
Soil	8	3	11	16.9%
Sand	10	2	12	18.4
Stone	4	1	5	7.6
Stick	10	1	11	16.9%
Total	**50**	**15**	**65**	**100%**

FIGURE – 5

DISTRIBUTION OF CORNEAL ULCER AMONG TRAUMATIC CASES

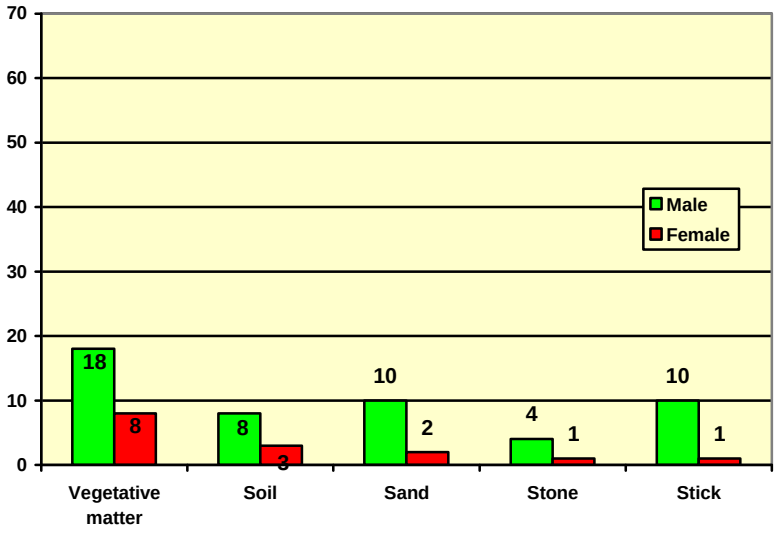
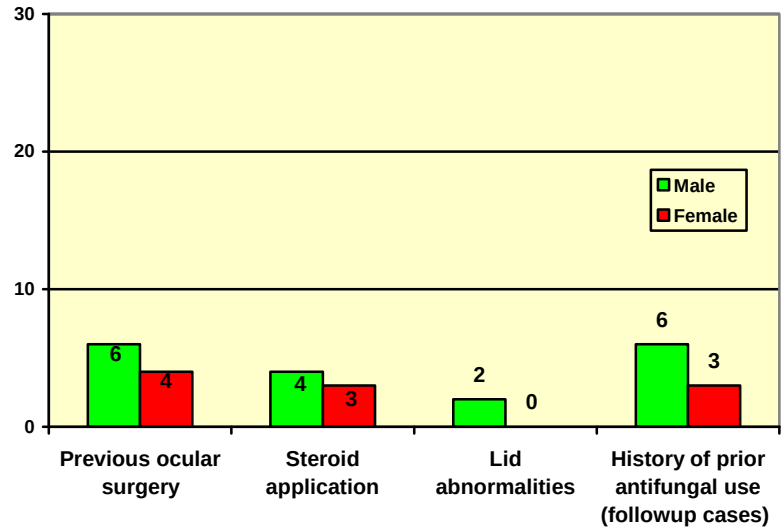


FIGURE – 6

DISTRIBUTION OF PREDISPOSING FACTORS OTHER THAN TRAUMA



Co-existing ocular diseases such as lid abnormalities, previous ocular surgery, steroid application and history of prior antifungal agents contribute to 3.63%, 18%, 12.7% and 16.36% respectively.

TABLE – 6

DISTRIBUTION OF PREDISPOSING FACTORS OTHER THAN TRAUMA

Non Traumatic origin	Male	Female	%
Previous ocular surgery	6	4	18%
Steroid application	4	3	12.7%
Lid abnormalities	2	0	3.63%
History of prior antifungal use (followup cases)	6	3	16.36%

The relationships of influence of various predisposing factors on the isolation of corneal pathogens were shown in table 5 & 6.

In analyzing the contribution of different trauma lesions in corneal ulcer, trauma with vegetative matter like paddy, leaf and wood were responsible for 40% of cases

TABLE – 7

DISTRIBUTION OF CORNEAL ULCER AMONG TRAUMATIC CASES

N = 120

Nature of Trauma	Male	Female	Total	%
Vegetative matter	18	8	26	40%
Soil	8	3	11	16.9%
Sand	10	2	12	18.4
Stone	4	1	5	7.6
Stick	10	1	11	16.9%
Total	**50**	**15**	**65**	**100%**

Among the bacterial isolates, *S.Pneumoniae* 9/22 (40%) was the Predominant organism followed by *Pseudomonas* 7/22 (31%), *Nocardia* 4/22 (18%) and *S.viridans* 2/22(9.09%).

TABLE – 8

DISTRIBUTION OF BACTERIAL AGENTS CAUSING CORNEAL ULCER

Total 22

Bacterial Isolate	Total No. of Isolate	%
Strep.pneumoniae	9	40%
Pseudomonas	7	31%
Nocardia	4	18%
Strep.viridans	2	9.09%

Among the fungal isolates, 28 out of 53 (52.83%) cases were due to *Fusarium* species and next common agent isolated was *Aspergillus flavus* 16/53 (30%), *Aspergillus fumigatus* 4/53 (7.5%), *Aspergillus niger* 3/53 (5.6%) and *Bipolaris* 2/53 (3.7%).

TABLE – 9

DISTRIBUTION OF FUNGAL AGENTS CAUSING CORNEAL ULCER

Total 53

Fungal Isolate	Total No. of Isolates	%
Aspergillus flavus	16	30%
Aspergillus fumigatus	4	7.5%
Aspergillus niger	3	5.6%
Fusarium	28	52.83%
Bipolaris	2	3.7%

Fusarium species were the most common fungal agent isolated 52.83%

Antibacterial sensitivity testing was performed by Kirby-Bauer method with drugs such as Gatifloxacin, Tobramycin, Ceftazidime, Vancomycin and Cotrimoxazole. 80% of *S.Pneumoniae*, 85.71% of *Pseudomonas*, 75% of *Nocardia* and 50% of *S.viridians* were sensitive to Gatifloxacin.

88% of *S.Pneumoniae*, 71% of *Pseudomonas*, 50% of *Nocardia*, and 50% of *S.viridans* were sensitive to Tobramycin. 55.55% of *S.Pneumoniae*, 42.85% of *Pseudomonas*, 50% of *Nocardia*, 50% of *S.viridans* were

FIGURE – 7

DISTRIBUTION OF BACTERIAL AGENTS CAUSING CORNEAL ULCER

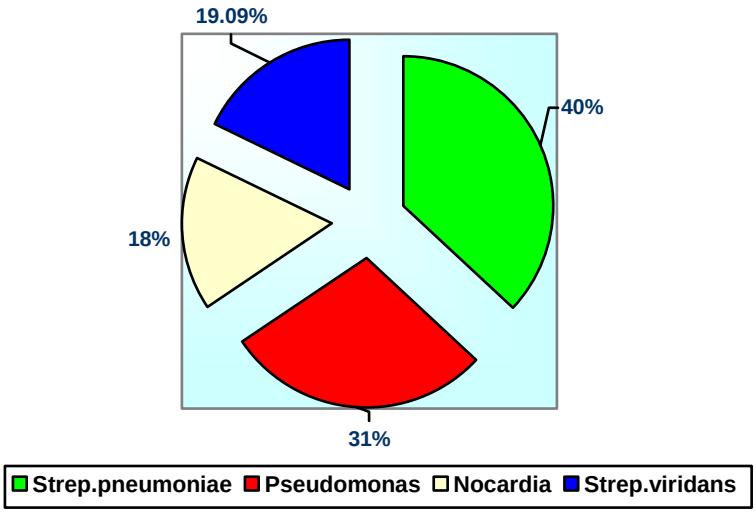
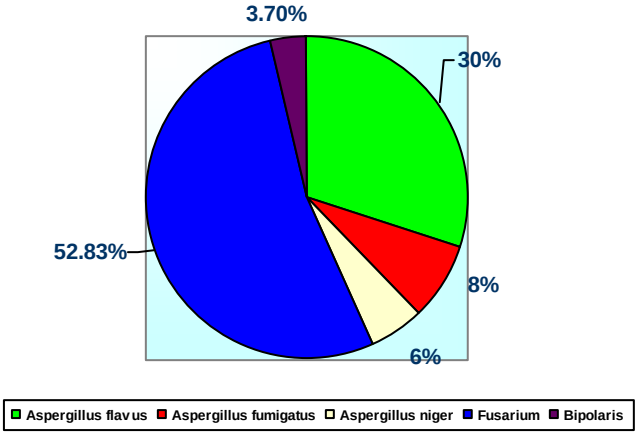


FIGURE – 8

DISTRIBUTION OF FUNGAL AGENTS CAUSING CORNEAL ULCER



sensitive to ceftazidime. All the 4 species were sensitive to vancomycin and 22.2% of *S.Pneumoniae*, 42.8% of *Pseudomonas*, 50% of *Nocardia*, and 50% of *S.viridans* were sensitive to Cotrimoxazole.

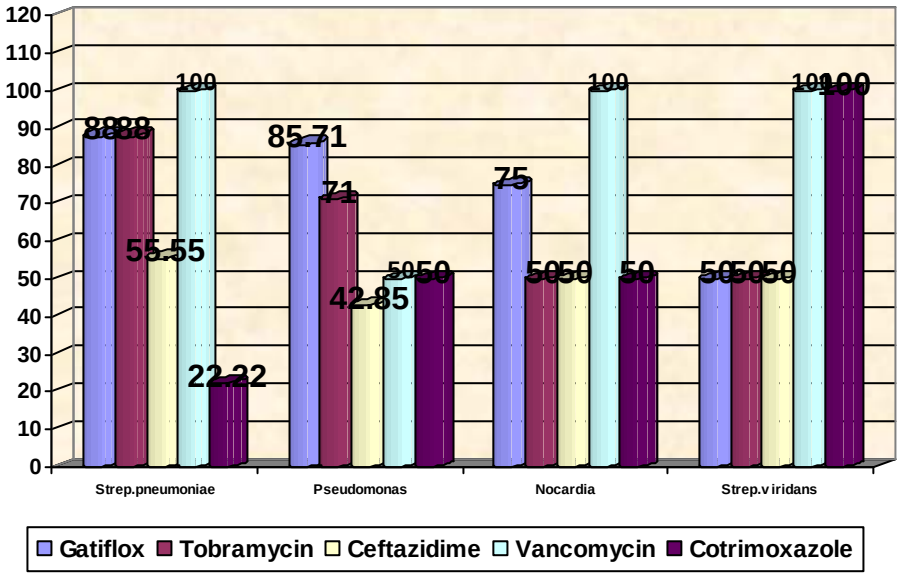
TABLE – 10

ANTI BACTERIAL SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES

Organism	Gatiflox	Tobramycin	Ceftazidime	Vancomycin	Cotrimoxazole
Strep.pneumoniae	8(88%)	8(88%)	5(55.55%)	9(100%)	2(22.22%)
Pseudomonas	6(85.71%)	5(71%)	3(42.85%)	7(100%)	3(42.8%)
Nocardia	3(75%)	2(50%)	2(50%)	4(100%)	2(50%)
Strep.viridans	1(50%)	1(50%)	1(50%)	2(100%)	1(50%)

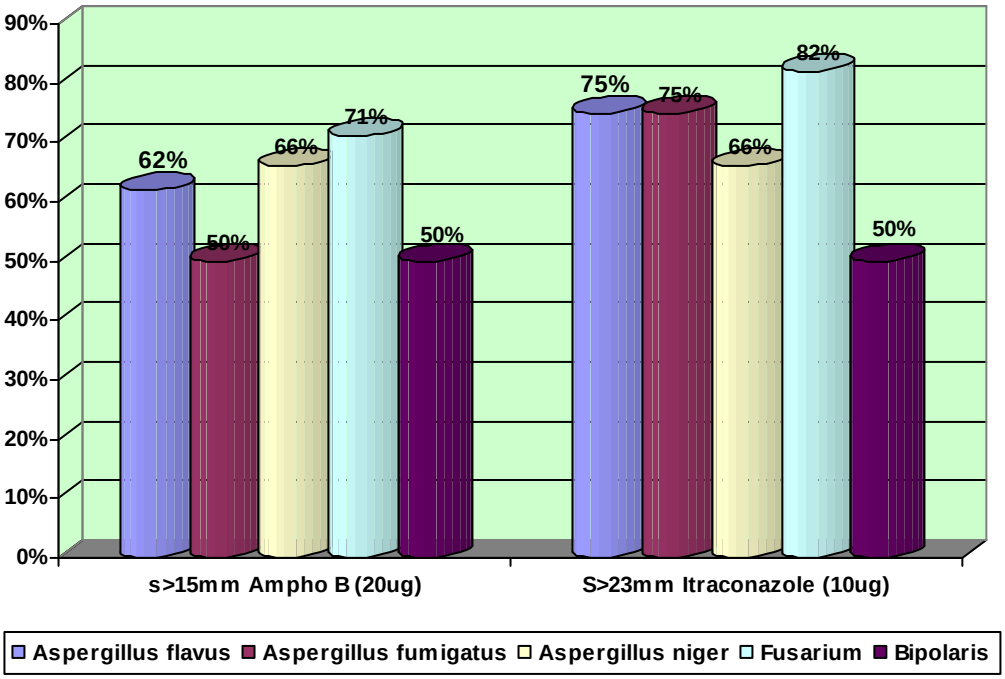
FIGURE –9

ANTI BACTERIAL SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES



FIGURE– 10

ANTI FUNGAL SUSCEPTIBILITY PATTERN OF FUNGAL ISOLATES



Antifungal susceptibility pattern of fungal isolates by Disc diffusion method shown 62% of *A. flavus*, 50% of *A.fumigatus*, 66.6% of *A. niger*, 82% of *Fusarium* and 50% of *Bipolaris* were sensitive to Amphotericin B.

TABLE – 11

ANTI FUNGAL SUSCEPTIBILITY PATTERN OF FUNGAL ISOLATES (Disk Diffusion Method)

Organisms	No. of Isolates	S > 15mm Ampho B (20ug)	S>23mm Itraconazole (10ug)	Flucanazole
Aspergillus flavus	16	10(62%)	12(75%)	0
Aspergillus fumigatus	4	2(50%)	3(75%)	0
Aspergillus niger	3	2(66%)	2(66%)	0
Fusarium	28	20(71%)	23(82%)	0
Bipolaris	2	1(50%)	1(50%)	0

75% of *A. flavus*, 75% of *A. fumigatus*, 100% of *A.niger*, 82% of *Fusarium* and 100% *Bipolaris* were sensitive to Itraconazole.All the fungal isolates 100% of the *(Aspergillus* species, *Fusarium* and *Bipolaris)* were resistant to Flucanazole.

MIC of Amphotericin B by agar dilution method showed MIC of less than 2 micro gram/ml. for all the *Aspergillus* species. *Fusarium* species showed MIC of less than 2 micro gram in 100% isolates. *Bipolaris* showed 100% sensitive range for Amphotericin B.

TABLE – 12

MIC OF AMPHOTERICIN B BY AGAR DILUTION METHOD

Organisms	**.625ug**	**0.125ug**	**.25ug**	**.5ug**	**1ug**	**2ug**	**4ug**	**8ug**
Aspergillus flavus			6	4	2	4		
Aspergillus fumigatus			2	2				
Aspergillus niger				3				
Fusarium		10	12	6				
Bipolaris			1	1				

MIC of Itraconazole by agar dilution method, all the isolates showed high sensitive range compared with Amphotericin B. all the 100%

Aspergillus, *Fusarium* and *Bipolaris* species showed MIC of less than 2 micro gram/ml for Itraconozole.

TABLE – 13
MIC OF ITRACONAZOLE BY AGAR DILUTION METHOD

Organisms	**.625ug**	**0.125ug**	**.25ug**	**.5ug**	**1ug**	**2ug**	**4ug**	**8ug**
Aspergillus flavus		3	5	4	3	1		
Aspergillus fumigatus			2	2				
Aspergillus niger				2				
Fusarium		10	15	3				
Bipolaris				2				

MIC determination by broth microdilution method also showed that the MIC range was comparable with agar dilution method. Good correlation was observed between agar dilution and broth microdilution method.

TABLE – 14

MIC OF AMPHOTERICIN B BY MICRO DILUTION METHOD

Organisms	**.625ug**	**0.125ug**	**.25ug**	**.5ug**	**1ug**	**2ug**	**4ug**	**8ug**
Aspergillus flavus			6	7	2		1	
Aspergillus fumigatus			2	1			1	
Aspergillus niger				3				
Fusarium		8	10	8	2			
Bipolaris			1	1				

TABLE – 15

MIC OF ITRACONAZOLE BY MICRO DILUTION METHOD

Organisms	**.625ug**	**0.125ug**	**.25ug**	**.5ug**	**1ug**	**2ug**	**4ug**	**8ug**
Aspergillus flavus		4	6	2	2		2	
Aspergillus fumigatus			2	1			1	
Aspergillus niger				3				
Fusarium		4	10	8	2	4	4	
Bipolaris				2				

TABLE – 16

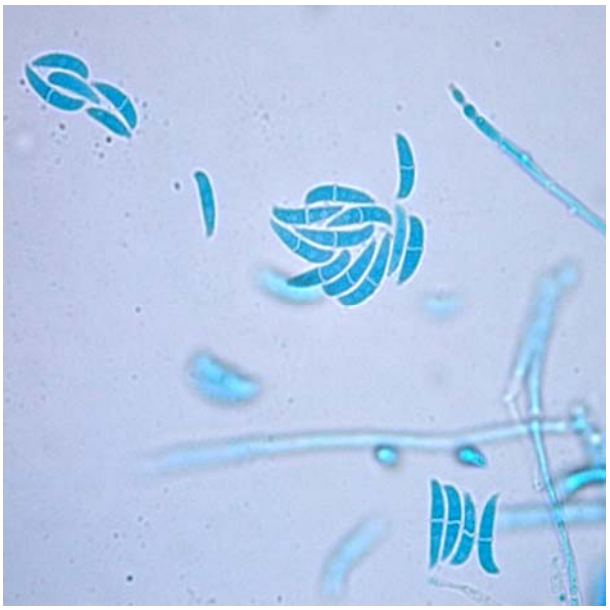
COMPARISON OF MIC IN AGAR DILUTION AND BROTH MICRO DILUTION

Drug Conc	Ampho B MIC < 2ug		Itraconazole MIC < 2ug	
Org	**Agar Dilution**	**Broth Micro Dilution**	**Agar Dilution**	**Broth Micro Dilution**
Aspergillus flavus	16	15	14	14
Aspergillus fumigatus	4	3	3	3
Aspergillus niger	3	3	2	3
Fusarium	24	24	25	25
Bipolaris	1	1	2	2

A good correlation was observed between agar dilution method and broth micro dilution method in the sensitivity pattern of fungal isolates with anti fungal drugs.

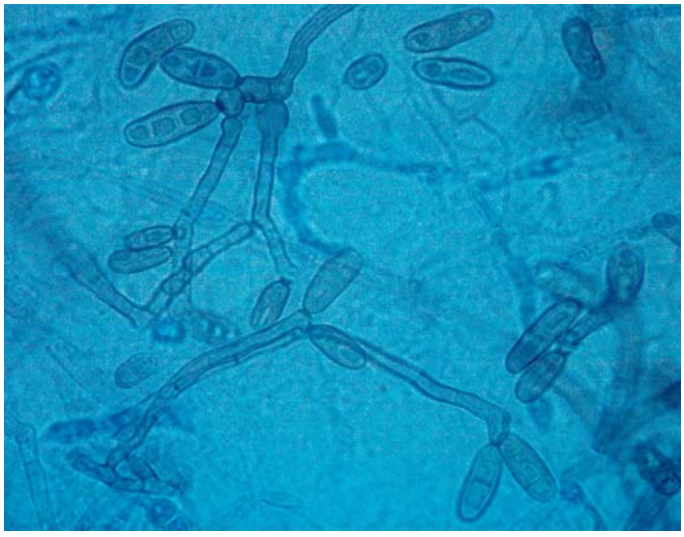
The correlation coefficient between agar diluition and micro dilution by using drug such as Amphotericin B and Itraconazole MIC < 2ug is .98 and .99 .

Fusarium (Macroscopic)

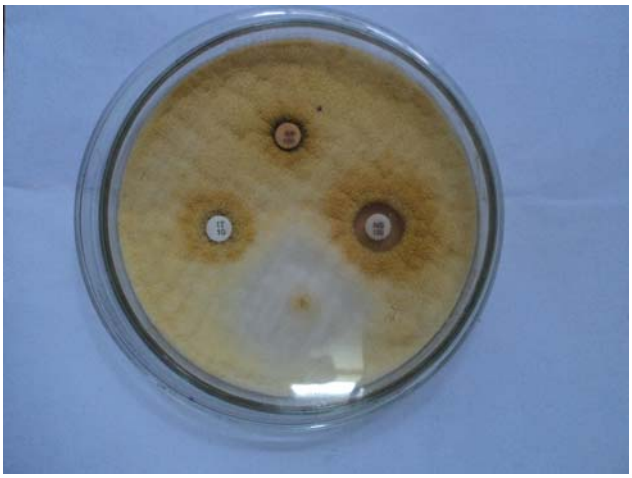


Fusarium (Microscopic)

Bipolaris (Macroscopic)



Bipolaris (Microscopic)



Antifungal sensitivity testing

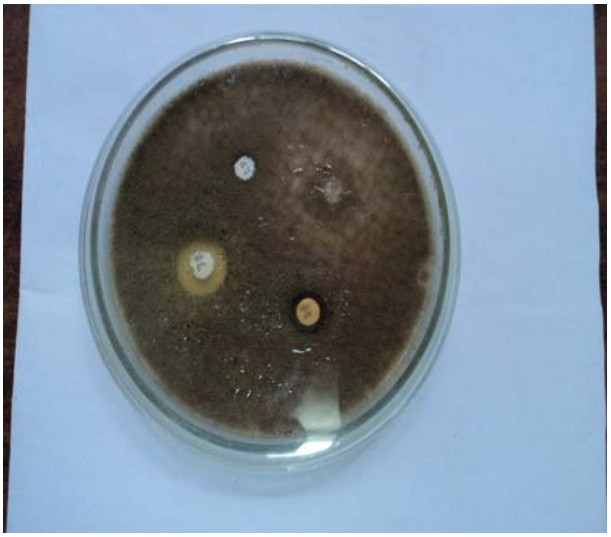
disk diffusion method

(Aspergillus flavus)

Antifungal sensitivity testing

disk diffusion method

(Aspergillus fumigatus)



Antifungal sensitivity testing

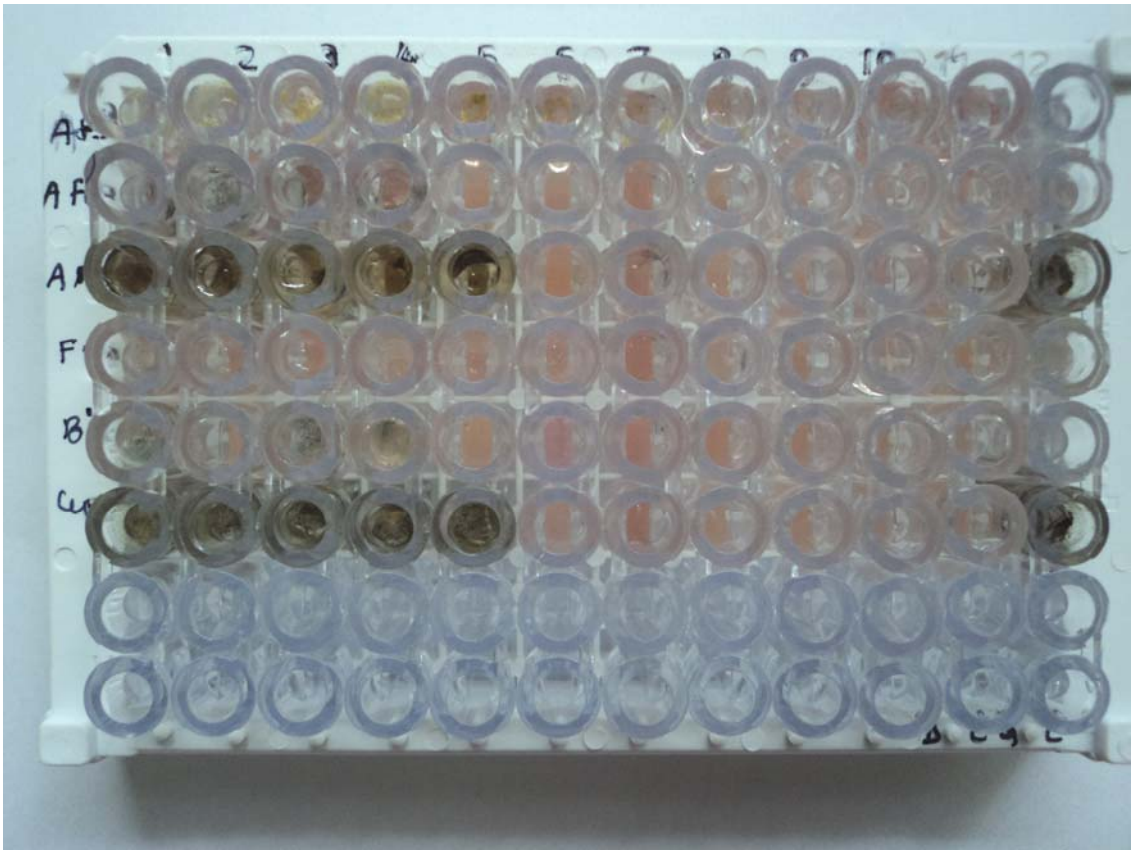
disk diffusion method

(Aspergillus niger)

Antifungal sensitivity testing

(agar dilution method)

RPMI -1640 medium



Antifungal sensitivity testing (micro dilution method)

DISCUSSION

Corneal ulcer is one of the most vision threatening ocular infection, which can cause significant morbidity. Patients can have poor clinical outcome including blindness if aggressive (or) prompt therapy is not initiated.

There have been numerous studies both in India and abroad on infectious corneal ulcerations in the past 20 yrs. In all these studies it has been observed that there is a changing spectrum of agents involved and predisposing factors in different geographical regions.

Microbial keratitis from either bacterial (or) fungal (or) parasitic infection exists in all geographic regions of the world. The entire population is at risk of developing corneal infection, but some are at greater risk than others.

The present study showed the following results. Out of 120 corneal ulcers studied in detail, 75 cases showed culture positivity which accounts for 62.85% of the patients. This study is nearly similar to the study of **M.Srinivasan et al**[97] in 1997 from Madurai which revealed (68.4%) positivity and **Geetha K.V et al**[29] in 2002 which showed 78% culture

positivity, Where as the study done by **sadia sethi et al** [89]at Peshawar in 2010 revealed 22% culture positivity. This shows that if proper culture techniques are followed , the percentage of culture positivity can be increased to 60%

In this study, male predominated females in all forms of keratitis. In this study, 77/120 (64%) were males and 43/120 (35.8%) were females. This study correlates well with the study of **sadia sethi et al**[89] in 2010 from Peshawar who reported 67% males and 33% females with corneal ulcer and **Reema nath et al**[86] in 2011 from upper Assam revealed increased incidence of corneal ulcer in male patients (67.6%). Corneal infection among males could be attributed to their greater involvement in out door activities, thus being prone to corneal injuries with external agents.

The distribution of corneal ulcer cases in rural and urban area revealed high prevalence of infected corneal ulcers in rural area70% . This study is concordant with study by **Basak samar et al**[90] in 2005 from West Bengal who reported that 78.5% of the patients were from rural areas.

In this study a very high percentage of the patients with history of corneal injury were recorded in patients with fungal keratitis 61%, where as in bacterial keratitis, corneal injury was found to be very low 16%. This

study correlates with the study of **Reema Nath at al** [86] in 2011 from Assam which revealed (74.5%) of keratitis due to trauma, but the study conducted by **sadia sethi et al**[89] in 2010 from Peshawar reported trauma contributed to 39% of keratitis cases.

In South India, paddy (or) rice stalks in the fields, thorns and tree branches were the most common cause of corneal injuries. In this study, corneal injury with vegetative matter contributes to 40%. This study correlates with the study concluded by **Basak Samar et al**[90] in 2005 West Bengal who reported (59.6%) traumatic cases due to vegetative matter. But, **M. Srinivasan et al**[97] in 1997 from Madurai, South India reported that the contribution of vegetative matter in causing corneal infection was 25.2%.

This difference in pattern of risk factors may be due to the variation in the occupational profile of the patients who live in those regions. Agricultural related works are common in developing countries.

Butler et al[12] in 2005 from Philadelphia reported that chronic ocular surface disease (25%), contact lens wear (20%) and use of topical corticosteroids (14% were common risk factors among non traumatic causes). But in this study, 16.36% of cases gave history of prior topical antifungal use, history of steroid application in 12.7%, history of prior ocular

surgery in 18% of the patients.

This study correlates with the study by **wong et al**[118] in 2003 from Newzealand who reported 29.6% of cases due to previous ocular surgery and history of steroid application in 15% of the patients.

In this study, bacteria and fungi were isolated in 60.8% samples out of this 64.38% were fungal isolates and 35.62% were bacterial isolates. Where as the study conducted by **MR.Kursiah et al**[54] in 2008 from Malaysia reported that 36% of fungi and 64% of bacteria were positive in culture. But the study concluded by **M.Srinivasan et al**[97] in 1997 from Madurai reported 47.1% of bacterial isolates and 46.8% of fungal isolates.

Among the bacterial isolates in this study 9(40%) were *S. pneumoniae,* followed by *Pseudomonas* 7(31%) and *Nocardia* 4(18%) and *S.viridans* 2(9.09%).

Among the Fungal, isolates, in this study *Fusarium* spp were 28/53 (52.8%), followed by *A. flavus* 16/53 (30.1%), *A. fumigatus* 4/53 (7.5%), *A.niger* 3/53 (5.6%) and *Bipolaris* 2/53 (3.7%)

This study correlates with the study by **M.Srinivasan et al**[97] from Madurai in 1997 who has reported that *S.Pneumoniae* (44.3%) was the most common bacteria followed by *Pseudomonas* spp (14.4%) and the most

common fungal pathogen isolated was *Fusarium* spp (47.1%) followed by *Aspergillus* spp (16.1%)

Feilmeier et al[24] from Nepal in 2010 has reported that among the fungi, *Aspergillus* was the most common organism and *S.pneumoniae* was the most common bacteria identified.

Samar K Basak et al[90] from West Bengal in 2005 reported that *Aspergillus* spp (60%) and *Staphylococcus aureus* (46%) were the most common fungus and bacteria respectively.

In a study from upper Assam by **Reema nath et al**[86] in 2011 has reported that *Fusarium* was the most common species isolated from corneal ulcer.

Sadia sethi et al[89] in 2010 from Peshawar reported that *Pseudomonas* is the predominant organism causing bacterial keratitis.

Gram stain examination of the corneal scrapings and 10% potassium hydroxide mount were analysed in evaluating the screening tests for rapid diagnosis of aetiological agents in infectious corneal ulcers.

10% KOH mount examination showed a sensitivity of 96% and specificity of 96% This study is similar to the study of **Vaj payee RB et al** [111] in 1993 which showed 94.3% sensitivity of 10%

KOH examination.

Although, culture of microbial organisms is considered to be the gold standard, direct microscopic examinations of smear provides immediate information about the causative organisms and helps in early initiation of treatment.

In the present study, antibacterial sensitivity test was performed by Kirby –Bauer's disc diffusion technique on Mueller Hinton agar plates. The antibiotics such as Gatifloxacin, Tobramycin, Ceftazidime, Vancomycin and Cotrimoxazole were used.

Antibacterial susceptibility pattern of bacterial isolates by Kirby-Bauer method showed that 88% *S. pneumoniae* spp, 85.7% of *Pseudomonas*, 75% of *Nocardia* and 50% of *S.Viridans* were sensitive to Gatifloxacin, 88% of *S. pneumoniae* and 71% of *Pseudomonas*, 50% of *Nocardia* and 50% of *Strep Viridans* were sensitive to Tobramycin. 55.55% of *S.Pneumoniae*, 42.8% of *Pseudomonas*, 50% of *Nocardia* and 50% *Strep.Viridans* were sensitive to Ceftazidime.

All the bacterial isolates were (*Strep pneumoniae, Pseudomonas, Nocardia* and *Strep.Viridans*) were 100% sensitive to Vancomycin.

This study is similar to the study of **Cesar Espiritu et al**[13] in 2008 from

Philippines which revealed 70% sensitivity of *S.pneumoniae* to Tobramycin, 84% sensitivity to fluroquinalone and 100% sensitivity to vancomycin.

Antifungal susceptibility pattern of fungal isolates done by disk diffusion method showed that 71% of *Fusarium*, 62% of *A.flavus*, 50% of *A.fumigatus*, 66.6% of *A.niger*, 50% of *Bipolaris* were sensitive to Amphotericin B. 82% of *Fusarium*, 75% of *A. flavus*, 75% of *A. fumigatus* and 66.6% of *A.niger* and 50% of *Bipolaris* were sensitive to Itraconazole.

All the organisms were resistant to Flucanazole by disc diffusion method.

MIC values of Amphotericin B. by agar dilution method for 4 fungal species were as follows. 20/28 (71%) of *Fusarium* species, *A.flavus*, 14/16 (87.5%) and *A.fumigatus* 3/4 (75%) showed MIC value of 2 micro gram per dl. and 50% (1/2 *Bipolaris* species showed the MIC value of less than 2 micro gram per dl.

MIC of Itraconazole by agar dilution method 25/28 (71%) of *Fusarium* species, 14/16(87.5%) of *A.flavus*, 2/3 (75%) of *A.fumigatus* and 2/2 100% of *Bipularis* species were sensitive to MIC range of less than 2 micro gram per dl.

MIC determination by broth micro dilution method showed similar

range of MIC when compared to agar dilution method.

The present study is similar to the study done by **Usha Arora et al** [107]in 2006 from Amristar who reported that >81% of *Aspergillus* species were resistant to Flucanazole and **Pankaj K Agarwal et al**[78] in 2001 from Calcutta whose study revealed that itraconazole is more effective in treating corneal ulcer. (more than 80% of fungi) were sensitive to itraconazole.

Both agar dilution and micro dilution showed good correlation.

KL Therese et al[102] in 2006 from Chennai has reported that *A.niger* exhibits high degree of resistance to Amphotericin B.

The reports of CLSI Broth dilution and agar dilution were comparable, indicating the suitability of the agar dilution method.

The CLSI broth micro dilution method (M-27a) is time consuming, expensive, and technically difficult to perform. On the other hand, the agar dilution method has an important advantage over the CLSI method. The advantage is the visual reading based on the intensity of growth showing the clear end point of inhibition.

The emergence of antifungal resistance has made susceptibility testing important though the applicability of invitro antifungal sensitivity testing may not directly correlate well with the clinical outcome.

CONCLUSION

Keratitis is more common during 3rd to 5th decade of life with male predominance and rural background.

Keratitis is more common during the paddy harvesting season.

Rural agricultural workers were more vulnerable to fungal Keratitis.

A variety of microbial organisms can produce infectious corneal ulceration. Among the bacterial isolate, S. pneumoniae was the most common organism and among the fungal isolate, Fusarium was the most common organism to produce keratitis.

Among the various predisposing factors, trauma in agriculturist plays an important role in producing corneal ulceration.

Diagnostic corneal scraping and culture (Gold standard) are mandatory in order to identify the causative organisms when infective keratitis is suspected and to choose appropriate antimicrobial therapy.

The present study indicates that the agar dilution method can be adopted for invitro antifungal sensitivity testing as it is simple, reproducible, cost effective and easy to perform technique in a routine clinical microbiology laboratory.

The increased incidence of fungal keratitis, coupled with a decreased bioavailability of donar corneas in developing countries, warrants further study of risk factors, antifungal susceptibility testing and possible pharmacologic combinations to prevent blindness.

SUMMARY

A Total of 120 cases of infectious keratitis were included in the study. Aetilogical organisms were isolated in 75 (62.85%) of the cases. 70.66% of them were fungi and 29.33% of them were bacteria. Majority of the organisms were fungi belonging to the genus Fusarium (52.83%) followed by Aspergillus spp (A. flavus 30%), (A. fumigatus 7.5%) and (A. niger 5.6%)

Male predominance was seen in this study (64.38%)

The age group most commonly affected was between 30-60 years constituting 58.3% of cases.

The incidence of infectious keratitis was more in rural area than in urban area 70% .

Among the predisposing factors, trauma with vegetative matter 40% was found to be more important in the development of infectious fungal corneal ulcers.

10% KOH mount found to be very sensitive rapid screening tests to diagnose fungal corneal ulcer with sensitivity of 96 %.

Fungal keratitis (70.6%) was more common than bacterial keratitis (29.3%)

Among the fungal isolates Fusarium species were the most common Fungal isolate and it accounts for 52.83%.

Among the bacteria S. pneumoniae was the most common organism followed by Pseudomonas (31%), Nocardia (18%) and S. viridans (9.09%)

88% of the bacterial isolates were sensitive to Gatifloxacin, 90% of the isolates were sensitive to Tobramycin, 55% of the Isolates were sensitive to ceftazidime. All were 100% sensitive to Vancomycin.

85% of the fungal isolates were sensitive to Amphotericin B. 90% of the fungal isolates were sensitive to Itraconazole. All the fungal isolates were resistant to Flucanazole by disk diffusion method.

Totally 85% of fungal isolates exhibited sensitivity range for Amphotericin B and 90% of the isolates exhibited sensitivity range for Itraconazole in agar dilution method.

In broth microdilution method, 80% of the isolates exhibited sensitivity range for Amphotericin B and 90% of the isolates showed sensitivity range for Itraconazole.

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APPENDIX – I

A. STAIN & REAGENTS :

1. GRAM STAIN :

Methyl violet (2%)	:	10g methyl violet in 100 ml absolute alcohol 1 lit. of distilled water (Primary stain)
Grams Iodine	:	10 g Iodine in 20 g KI (Fixative)
Acetone	:	Decolorising agent
Carbol fuschsin 1%	:	Counter stain

2. 10% KOH :

Potassium hydroxide	:	10 g
Glycerol	:	10 ml
Distilled water	:	80 ml

3. LACTOPHENOL COTTON BLUE:

For the staining and microscopic identification of fungi.

Cotton blue (aniline blue)	:	0.05 g

Phenol Crystals (C6H504)	:	20 g
Glycerol	:	40 ml
Lactic acid (CH3CHOHCOOH)	:	20 ml
Distilled water	:	20 ml

APPENDIX - II

SABOURAUD'S DEXTROSE AGAR :

Dextrose	:	20g
Neo Peptone	:	10g
Agar	:	20g
Distilled water	:	1000 ml

All the ingredients were dissolved in distilled water and dispensed in screw capped bottles and sterilized by autoclaving at 121 C for 20 minutes.

Note : Cycloheximide was not added to the media since it is known to inhibit ocular fungal pathogen.

MULLER HINTON AGAR

Beef extract	:	300 ml.
Caesein hydroxylate	:	17.5g
Starch	:	1.5g
Agar	:	10g

Distilled water : 1 liter

Starch was emulsified in small amount of cold water and beef extract , Caesein hydroxylate and agar were added and all these things were dissolved in distilled water to make the volume of 1 liter. The pH was adjusted to 7.4, dispensed in screw capped bottles and sterlised by autoclaving at 121 C for 20 minutes.

RPMI MEDIUM

Commercially purchased RPMI 1640 media supplemented with L Glumate without sodium bi carbonate. It was dissolved in nuclease free water and sterilized by filtering through a sterile membrane filter with a porosity of 0.22 microns.

PROFORMA

Name : Age : Sex : M/F

OP/IP No. Date of sample collection:

Occupation :

Place of work : Rural / Urban

Address : Socio economic status :

CORNEAL ULCER DETAILS

Affected Eye : R/L Duration :

Vision : RE : LE:

H/O Trauma : Y/N

History of

a. Hypertension : Y/N
b. Diabetes Mellitus : Y/N

History of Opthalmic surgery : If any specify

History of recent Antifungal use : Topical/oral/injectable

History of steroid use : Topical/oral/injectable

MICROBIOLOGICAL PROFILE

Gram stain :

Modified acid fast stain :

KOH mount :

LPCB mount :

Antibacterial sensitivity report : Sensitive / Resistant

Antifungal sensitivity : Sensitive / Resistant

Ref. No. 15806/ E4/3/2010

Govt. Rajaji Hospital, Madurai -20.
Dated 5.1.2011

Sub : Establishment – Government Rajaji Hospital, Madurai-20 – Minutes of the Ethical Committee meeting held on 9-12-2010 - Regarding.

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Minutes of the Ethical committee meeting held at 12.00 Noon on 09.12.2010 at the Medical Superintendent's Chamber, Govt. Rajaji Hospital, Madurai. The following members of the committee were present and the following points were decided in the meeting.

1.	Dr.S.M.Sivakumar, MS, Govt. Rajaji Hospital, Madurai	Medical Superintendent	Convenor
2.	Dr.N. Vijayasankaran, M.Ch(Uro)	Sr. Consultant Urologist, Madurai Kidney Centre, Sivagangai Road, Madurai	Chairman
3.	Dr.T.Meena, MD	Prof of Physiology Madurai Medical College	Member
4.	Dr. Moses K.Daniel MD	Professor of Medicine, Madurai Medical College	Member
5.	Dr.S.S. Dilsath, MD (O & G)	Prof. of Ob & Gyn Madurai Medical College	Member
6.	Dr.B.K.C. Mohan Prasad, M.Ch (Surg.Oncology)	Prof. of Surg. Oncology, Madurai Medical College	Member Secy
7.	Shri.S. Sivakumar, M.A(Social) M.Phil	Sociologist, Plot No. 51 F.F., K.K. Nagar, Madurai -20.	Member

The following Projects are Approved:

Sl.No.	Name of the Applicant	Course	Name of the Project	Remarks
1	Dr.Geetha Ramachandran	Scientist 'C" (Principal Investigator) Dept. of Clinical Biochemistry and Pharmacology	" Pharmacokinetics of anti-tuberculosis drugs in children:impact of age, nutritional status and HIV infection	Approved

2.	Dr. K. Nalini	Lecturer in Microbiology Dept., Ayya Nadar Janaki Ammal College, (Autonomous) Sivakasi	Molecular Analaysis of Multidrug Resistant Gene From Bacterial Isolates Associated with Urinary Tract Infections	Approved
3.	Dr.W. Isabel	P.G. Professor & Head, PG & Research Department of Zoology, Lady Doak College, Madurai	A study on the level of Biochemical Profile, certain elements & PKD genes Polymorphism in patients with Polycystic Kidney Disease in Madurai	Approved
4.	Dr.T.Rajendran	PG student in MD(Microbiology) Institute of Microbiology, MMC, Madurai	Comparison of the Efficacy of Disc Diffusion method with other methods – E Test, Oxacillin Resistance, screening Agar in detection of Methicillin Resistance staph, aureus.	Approved
5.	Dr.D.Saradha	PG student in MD (Micro biology) Institute of Microbiology, MMC Madurai	Aetiological Agents involved in Microbial Keratitis	Approved
6.	Mr.Praveen.J	II Year M.Sc. Nursing Student, CSI Jeyaraj Annapackiam College of Nursing, Pasumalai, Madurrai	Seeking permission to conduct the research study – A study to assess the effectiveness of chewing gum on early bowel movement after major abdominal surgeries in GRH, Madurai	Approved
7.	Dr.A. Mahalakshmi	Research Associate, C/o.Dr.P.Gunasekaran, Dept. of Genetics, School of Biological Sciences, MKU, Madurai	Developing a reference set of microbial genome sequence and preliminary characterization	Approved

			acebrophylline in patient with bronchial asthma	
25.	Dr. Vinay kumar Gurunath	PG Student, General Surgery, MMC Madurai	A Clinical Study on Typhoid Ileal Perforation	Approved
26.	Dr. A. Ramesh	PG Student, General Surgery, MMC Madurai	A Study of Incisional Hernia Incidence And Risk Factors.	Approved
27.	Dr. Aswin. K.	PG Student, General Surgery, MMC Madurai	A Clinical Study in Gastric Outlet Obstruction	Approved
28.	Dr. Kamalraj. R.	PG Student, General Surgery, MMC Madurai	A study of association between BPH and Inguinal Hernia	Approved
29.	Dr. Anoop. P.S.	M.S. P.G in E.N.T., MMC, Madurai	Endoscopic Findings and Radiological Apperance in Chronic Rhinosinusitis	Approved
30.	Dr. G. Selvarani	Assistant Professor, Cardiology, MMC, Madurai	A Study of Metabolic Syndrome, Obesity And Co-Morbidies in a Special Population (Staff Nurses)	Approved
31.	Dr. R. Chellapandian.	PG Student, General Surgery, MMC Madurai	Case Study of Abdominal Surgical Site Infection	Approved
32.	N. Jessie Metilda	Associate Professor, CSI Jeyaraj Annapackiam College of Nursing, Pasumalai, Madurai	A Study to asses the determinants and practice of permanent family planning method among eligible couples at Madurai District.	Approved

Medical Superintendent

MEDICAL SUPERINTENDENT
Govt. Rajaji Hospital,
MADURAI.

To : The Applicants

A STUDY ON MICROBIAL KERATITIS

Abstract:

Aims / Background:-

To determine the epidemiological characteristics and risk factors predisposing to corneal ulceration in and ground Madurai, south India and to identify the aetiological agents (bacteria and fungi) and their antimicrobial susceptibility pattern.

Materials and Methods:

It was prospective study performed at department of ophthalmology, institute of Microbiology, Madurai Medical college, Madurai and Aravind eye hospital, Madurai from December 2010 to July 2011. Patients diagnosed as infective corneal ulcers were included in the study. Age, Sex, cause of the ulcer and the results of scraping were recorded and their antimicrobial sensitivity pattern were analysed by standard microbiological techniques.

Results:

Out of 120 patients 77 (64.38%) were males and 43 (36%) were females. 50 (41%) were < 30yrs, and 70 (58.3%) were >30yrs of age. Ocular trauma was the most common cause found in 65 (54.16%) patients. 75 (62.5%) cases showed

positive culture. Among the bacteria, streptococcus pneumoniae was the most common organism isolated 9/22 (40%) and it was sensitive (88%) to Gatifloxacin and tobramycin and among fungi, Fusarium was the most common fungus isolated 28/53 (52- 83%) and it was sensitive to itraconazole and Amphotericin B.

Conclusion

Cooneal infections are more common in middle aged men. Ocular trauma is the leading cause. Streptococcus Pneumoniae is the most common bacteria and Fusarium is the most common fungus causing keratitis. Epidemiology of corneal ulcer is important in early initiation of empirical therapy.

[Key words : Keratitis, empirical therapy]